IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial Number:	10/081,955
Filed:	February 20, 2002
Applicants:	George E. Seidel, Lisa Herickhoff, John Schenk
Title:	System for Improving Yield of Sexed Embryos In Mammals
TC/A.U:	1634
Examiner:	Carla J. Meyers
Assignee:	XY, Inc.
Attorney Docket:	XY-Super-Cont2
Customer No.	33549

DECLARATION BY DAVID G. CRAN UNDER 37 C.F.R. §1.132

I, David G. Cran, Ph.D., declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

I am currently employed as the Director of Science at XY, Inc. My prior experience includes 17 years at the Animal Research Station and the Babraham Institute, each in Cambridge, UK, engaged in work related to animal reproduction. Such work has included extensive study of animal reproduction processes, including the physiology and structure of eggs, spermatozoa, and the fertilization process in bovine animals. Moreover, I was involved in early studies on sperm sorting and operated a Confidential Scientific Agreement concerning the development of this technology between my company in Cambridge, UK and the USDA in Maryland, USA from about 1991 to 1994. My work has included running the project which resulted in the births of the first sex selected cattle by IVF.

My duties require me to be extensively familiar with practices and developments in the field of animal reproduction. Accordingly, I have at various times reviewed various technologies related to this field. Such review has included the review of relevant academic and scholarly literature, my own empirical experimentation, and extensive discussion and collaboration with other researchers in the field. I have authored and co-authored several publications reviewing the state of the art in this field, including: Cran D.G., "XY Sperm Separation and Use in Artificial Insemination and Other Arts", Soc. Reprod. Fertil. Suppl., 2007, 65:475-91; De Graaf S.P., Evans G., Maxwell W.M., Cran D.G., O'Brien J.K., "Birth of Offspring of Pre-Determined Sex After Artificial Insemination of Frozen-Thawed, Sex-Sorted and Re-Frozen-Thawed Ram Spermatozoa", Theriogenology, 2007, 67:391-8; Lu K.H., Cran D.G., Seidel G.E. Jr., "In Vitro Fertilization With Flow-Cytometrically-Sorted Bovine Sperm", Theriogenology, 1999, 52:1393-405.

I am aware of and have reviewed the superovluation example described on pages 22-23 of U.S. Patent Application 10/081,955, filed February 20, 2002, entitled "System for Improving Yield of Sexed Embryos In Mammals", said pages attached to this Declaration as Exhibit "A" (the

"Superovulation Example"). I also am aware of and have reviewed the low dose discussion on page 19 of U.S. Patent Application 10/081,955, filed February 20, 2002, entitled "System for Improving Yield of Sexed Embryos In Mammals", said pages attached to this Declaration as Exhibit "B" (the "Low Dose Discussion").

With regard to the Superovulation Example, the fertilization success rates shown to have been achieved were for sorted sperm. It is generally accepted in the field of sex selection that sorted sperm are more difficult to work with than unsorted sperm. This is because working with sorted sperm requires additional steps not required for working with unsorted sperm, such as the sorting step itself, for example by flow cytometery, as well as any additional attendant steps, for example extending the sperm, concentrating the sperm, and the like. These additional steps may tend to complicate the artificial insemination process and even may tend to compromise the fertilization effectiveness of sperm. Because sorted sperm generally are more difficult to work with than unsorted sperm, fertilization success rates achieved with sorted sperm generally may be presumed to be capable of being achieved with unsorted sperm. For this reason, it may be presumed that had unsorted sperm been used in the Superovulation Example, the fertilization rates achieved would have been at least as successful as was the case for the sorted sperm actually used.

With further respect to the Superovulation Example, a fertilization success rate of at least 68% may be assumed because a total of 96 embryos and unfertilized oocytes from 9 of 12 inseminated heifers were recovered. Of those, 52 were embryos at normal stages of development and 13 were retarded embryos. Thus, 65 out of a total of 96 were fertilized, or 68%.

With respect to the Low Dose Discussion, I would understand the sentences reading "Typical artificial insemination is presently conducted with millions of sperm for bovine species..." and "For bovine sperm where currently 1 to 10 million sperm are provided..." as intending to communicate the concept that a typical unsorted insemination dosage for bovine animals would encompass the range of 1 to 10 million sperm. This understanding is based on my reading of this text as a person having at least ordinary skill in the art of bovine artificial insemination, and is based on the context of the paragraph in which these sentences appear as well as the plain meaning of the sentences themselves.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 12th day of February, 2008.

David G. Cran, Ph.D.

Exhibit A

reported to potentially achieve enhanced fertility in heifers as discussed in the article "Prostoglandin $F2_{\alpha}$ - A Fertility Drug in Dairy Cattle?", 18 Theriogenology 245 (1982) hereby incorporated by reference. While recent results have not maintained this premise, it may be that the present invention demonstrates its particular viability in situations of sexed, low dose insemination. For bovine species, artificial insemination may then be accomplished through the use of embryo transfer equipment with placement of the sperm cells deep within the uterine horns. This may be accomplished not at the peak moment as typically used in artificial insemination, but rather at a somewhat later moment such as 12 hours after that time since there is some possibility that fertility for sexed artificial insemination may occur slightly later. The utilization of embryo transfer equipment may be used because there may be high sensitivity of the uterine wall for such low dose, sexed inseminations.

Further the techniques can be combined to achieve higher efficiency production as well. Particularly, the processes now invented which permit high speed sorting and low dose insemination of sexed embryos is also possible in a superovulated animal. The superovulation may be achieved by use of a superovulatory pharmaceutical or by any other technique. The superovulatory pharmaceutical may act directly or indirectly, such as through a sequence of reactions to achieve a greater than normal production of eggs. The combination with superovulation is surprising because superovulation was previously deemed to hinder such a combination. Sperm transport is compromised in superovulated cattle, so, animals were frequently artificially inseminated on multiple occasions and/or with multiple doses of semen. Also, prior procedures for sexing semen were relatively slow; therefore, it was of interest to determine fertilization rates after a single insemination of superovulatory pharmaceutical, such as FSH (follicle stimulating hormone)-treated cattle with only 600,000 total sexed unfrozen sperm using these newer combination of techniques.

By example, twelve Angus crossbred heifers were superovulated using standard procedures: 6, 6, 4, 4, 2, 2, 2, and 2 mg FSH were injected intramuscularly at half-day intervals beginning between days 9 and 12 of the estrous cycle; 25 and 12.5 mg prostaglandin F-2 alpha were injected intramuscularly with the 6th and 7th FSH injections. Sperm from bulls of unknown fertility were

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stained with Hoechst 33342 and then sorted using a MoFlo® flow cytometer/cell sorter yielding 700-800 live sperm of each sex/sec. Average sort purity was 89% of the desired sex. Sorted sperm were concentrated to 3.36 x 10^6 sperm/ml by centrifugation at 650 g for 10 min, cooled to 5° C, and stored 4h. Then 184 ul were loaded in 0.25 ml plastic straws; half the dose was inseminated into each uterine horn 20 to 24 h post-onset of estrus using automatic side-opening embryo transfer sheaths. Embryos were collected by standard non-surgical procedures at 7 or 16 days post-estrus. Results were similar between day 7 and 16 collections and between X- and Y-sorted sperm. Embryos were recovered from 9 heifers. There were 52 embryos (mean, 4.3±5.3/donor) at normal stages of development, 13 retarded embryos and 31 unfertilized ova. Forty-six embryos were sexed by PCR using primers for a Y-chromosome-specific DNA sequence; 43 (93%) were of the intended sex. Although this study involved few animals, surprisingly, insemination of superovulated heifers with only 600,000 total (live) sexed unfrozen sperm gave similar results to conventional procedures. Variations on the above may also be accomplished, including, but not limited to, sorting through other than flow cytometric means, achieving superovulation in other manners, increasing fertility in other manners, and the like.

Further, the congruence of methods of sexing sperm based on DNA content, high speed flow cytometer/cell sorters, and procedures for inseminating heifers with fewer than 500,000 total sperm without compromising fertility has resulted in the possibility of a viable sexed semen industry in cattle within a few years. There will be a myriad of applications for sperm sexed at <85% accuracy. Perhaps the most obvious is inseminating one subset of cattle (both dairy and beef) for female herd replacements, and having the converse subset (both dairy and beef) bred to entirely different types of bulls to produced males for meat. A very important subset of the above is inseminating heifers with X-chromosome-bearing sperm to produce female calves, which have a lower incidence of dystocia than male calves, primarily due to smaller size. Furthermore, proving young dairy sires would be much more efficient with a preponderance of heifer calves. Having more than 85% heifer calves also makes it feasible to manage dairy cows so they average fewer than two surviving calves per lifetime, which is attractive because of reducing problems associated with gestation and parturition. Single sex systems of beef production also would become feasible, in which each female replaces herself and is slaughtered between 2 and 3 years of age, thus using a much higher percentage of nutrients in the

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Exhibit B

Another aspect which may interplay in the various factors of the present invention is that of utilizing low dose amounts of sperm for artificial insemination or the like. Additional background on the aspect of sexed, artificial insemination may be found in "Prospects for Sorting Mammalian Sperm" by Rupert P. Amman and George E. Seidel, Jr., Colorado Associated University Press (1982) hereby incorporated by reference. As mentioned, natural insemination involves numbers of sperm on the order of billions of sperm. Typical artificial insemination is presently conducted with millions of sperm for bovine species and hundreds of millions of sperm for equine species. By the term "low dose" it is meant that the dosage of sperm utilized in the insemination event are less than one-half or preferably even less than about 10% of the typical number of sperm provided in a typical artificial insemination event. Thus, the term "low dose" is to be viewed in the context of the typical artificial insemination dosage or also as an absolute number. For bovine sperm where currently 1 to 10 million sperm are provided, a low dose process may be considered an absolute number of about 500,000 sperm or perhaps as low as 300,000 sperm or lower. In fact, through utilization of the techniques of the present invention, artificial insemination with good percentages of success has been shown with levels of insemination of sperm at 100,000 and 250,000 sperm (41% and 50%, respectively pregnancy rates). As shown in the article "Uterine Horn Insemination of Heifers With Very Low Numbers of Non-frozen and Sexed Spermatozoa" as published in 48 Theriogenology 1255 (1997) hereby incorporated by reference. Since sperm cells appear to display a sensitivity to dilution, these results may display particular interdependence on the utilization of low dose sperm samples with regards to various techniques of the present invention. The absolute numbers may be species dependent, for equine species, merely less than about ten, five, or even one million sperm may be considered a low dose process.

Another aspect which may be important is the fact that the sperm sexed through the present invention techniques is utilized in an artificial insemination system. Thus, when the collector (14) is used to provide sperm for artificial insemination the techniques of the present invention may be particularly relevant. Further, it is possible that the combination of both artificial insemination use and the use in a low dose environment may together create synergies which makes the various

EXHIBIT B TO RESPONSE

SUPEROVULATION IN CATTLE: FROM UNDERSTANDING THE BIOLOGICAL MECHANISMS TO GENOMICS OF THE OOCYTE

DIELEMAN, S.J.

Department of Farm Animal Health, Faculty of Veterinary Medicine
Utrecht University, The Netherlands
E-mail: s.j.dieleman@vet.uu.nl

Abstract

Superovulation still constitutes the most widely used technique producing embryos in the cow for breeding but also to obtain large numbers of oocytes and embryos to investigate biological mechanisms in relation to competence of oocytes and embryos ultimately to develop into viable offspring. Of the variety of stimulation protocols that are applied for many decades those with a controlled release of the LH surge appear to be useful for research and practice.

The eCG/anti-eCG and a FSH protocol with norgestomet/GnRH-controlled LH surge are compared regarding effects on follicles, oocytes and embryos. In general, stimulation with gonadotropins also results in part of the oocytes and embryos being not competent or viable. Therefore, it is prerequisite to distinguish competent oocytes from non-competent. The steroid profile in the follicular fluid appears to provide reliable criteria assuming that functional preovulatory-sized primarily enclose competent oocytes.

Transcriptomics of the maturing oocyte have been studied following stimulation with gonadotropin using SSH and analysis by QPCR. The genes that were up-regulated at the start of resumption of meiosis related to different phosphodiesterases (PDE7), G-proteins, and regulators of G-protein signaling in meiotic resumption, molecular components involved in chromatids separation, regulation of Ca^{2+} oscillatory activity and cell cycle regulation. Most of these genes were identified for the first time in the mammalian oocyte. In addition, mRNAs were identified involved in correct translocation of organelles and segregation of chromosomes possibly explaining disturbed polar body emission and defects in cytoplasmic maturation as commonly observed in deviant oocytes from stimulated animals and in in-vitro matured oocytes. Finally, new information has been obtained regarding long chain fatty acid transport into the oocytes and regulation of energy requirement during maturation and blastocyst stage using lipid as a substrate, which may be used to adapt in-vitro culture of oocytes in many applications.

In conclusion, superovulation with a controlled LH surge is exceptionally useful to study regulation of oocyte competence in the cow but also as model for human IVF.

1. Introduction

Embryo transfer (ET) in cattle is used worldwide in cattle breeding for improvement of genetic quality and has recently been proposed anew to overcome fertility problems as for example caused by heat stress [1]. Although from the late 1980-ties on in-vitro produced embryos have been used successfully for ET superovulation still constitutes the major technique to obtain embryos [2,3]. Understanding the biology of treatment with

gonadotropins will not only improve application of superovulation in ET practice but also is essential to further knowledge on the basic mechanisms determining oocyte competence and embryo viability.

In follicles 2 to 3 mm in diameter the oocyte has acquired and stored most proteins and mRNA needed for further development and production of such compounds is largely terminated at this stage. Comparing the mRNA populations in oocytes collected from follicles < 2 mm to those from follicles > 5 mm [4] certainly contributes to identify genes involved in competence in broader sense. Following further development until (pre-) ovulation adds relatively few of these compounds. But they may be essential in determining the outcome of producing a competent oocyte to be fertilized and going through the first cell cycles until the transition from the maternal to the embryonic genome (MET) occurring between the 8- to 16-cell stage of early embryonic development. Global gene expression analysis during in-vitro maturation [5] will elucidate genes that are involved but may not pinpoint those that play a key-role in vivo. It appears, therefore, to be crucial to compare the transcriptome of in-vivo oocytes at onset of resumption of meiosis with that during final maturation using techniques such as suppression subtractive hybridization (SSH) which allows identifying up- or down-regulated low copy number mRNA transcripts.

It is evident that the single preovulatory oocyte and embryo of the untreated, normally cyclic cow remain the "golden" standard for any study on oocyte competence and embryo viability. However, to obtain for instance sufficient RNA simple calculation shows that approx. 1,000 cows/cycles are needed collecting round-the clock, while when using superovulation with a controlled LH surge some 50 cows will provide the required tissues and that during regular working hours. It is common knowledge that a substantial proportion of oocytes and embryos is not competent or viable following superovulation treatment, and although the sperm cell may contribute minute amounts of mRNA (See for review [6]) the origin of the oocyte determines the success of early embryonic development [7]. Therefore, discriminating follicles containing competent oocytes is a prerequisite to apply superovulation for research into topics such as nucleolar formation [8,9], metabolomics [10], mRNA expression [11], DNA methylation [12], proteomics [13], selective degradation of transcripts [14], and functional genomics [15] in relation to competence and embryo viability.

The aim of the present paper is to review briefly some superovulation protocols using eCG and FSH in relation to the effects on oocyte and embryo quality, and application of superovulation as a tool to study transcriptomics of competent oocytes.

2. Superovulation protocols

For many decades a variety of stimulation protocols with compounds containing FSH-activity have been used to collect large quantities of oocytes or embryos from the cow [16-18]. In general, the gonadotropin is administered at the onset of a follicular wave when FSH-activity is needed to start follicular development. This activity has to be present for several days to support further growth of the follicles, which is accomplished by gonadotropins with a long biological half-life such as eCG [19] or repeated administration of FSH preparations from ovine or porcine pituitary origin. Continued FSH-activity provides for signaling events at 2 different levels controlling the changes that must take place for follicular growth and attainment of oocyte developmental competence. The first signaling event comes from the

proper differentiation of the follicle as it normally occurs in the dominant follicle in preparation for ovulation. The second signaling event occurs as the process of follicle differentiation signals directly to the oocyte, possibly through the cumulus cells (See for review [20]).

The long-lasting eCG appears to have adverse effects on the competence of part of the oocytes possibly due to high estradiol concentrations in some of the preovulatory follicles affecting spindle formation [21] which can be suppressed by administering anti-eCG as originally developed by Bouters et al. [22]. It is evident that timing of the anti-eCG treatment in relation to the follicular development and the preovulatory LH surge is crucial [23]. When anti-eCG neutralized the FSH-activity before or at the onset of the LH surge the capacities to produce estradiol of the stimulated follicles was dramatically reduced, and release of the LH surge and thereby multiple ovulation did not occur in a majority of the animals. This makes the eCG/anti-eCG protocol practical primarily under laboratory conditions when e.g. the occurrence of the LH surge can be monitored using rapid radio immuno assay [24] facilitating anti-eCG administration shortly after the LH surge. Although almost 2-fold higher ovulation rates and yields of viable embryos were reported [24,25], in practice using fixed time protocols produced variable embryo yields (See for review [26]) leading to anti-eCG administration around ovulation which does not improve embryo yields but prevented formation of ovarian follicular cysts [27].

Following stimulation by gonadotropins, not all oocytes will show the same developmental competence due to deviations in preovulatory follicular development [28,29]. This heterogeneity in quality is probably due to intrinsic differences between oocytes originating from different follicular microenvironment as can be inferred from the considerable evidence for endocrine regulation changes after stimulation compared to normal cyclic cows. Firstly, a reduction occurs of endogenous basal secretion, pulse frequency and amplitude of FSH and of pulse frequency of LH by more than 50% [30,31], as well as a shortening of the period of preovulatory follicular development from 61 to 41 h in comparison to unstimulated cows [19]. Secondly, superovulation treatment has been shown to induce abnormal amounts of steroids in serum compared to the physiological levels seen during natural cycles [19,32-35]. Thirdly, different studies have also shown that follicular cells derived from stimulated cows have altered gonadotropin receptor mRNAs [36] and altered abundance of several transcripts for steroidogenic enzymes [37].

To explain and to improve the variability in oocyte competence to develop into viable embryos, the amount of required LH bioactivity in the follicle stimulating gonadotropin has been studied extensively. The eCG and FSH preparations with high LH bioactivity have been shown effectively to induce multiple follicle development, final oocyte maturation, ovulation and corpus luteum formation [38-40]. Currently, in clinical applications purified pituitary FSH is used either with added LH to a bioactivity ratio of 1:1 or with only a low remaining LH bioactivity, both products showing similar yields of viable embryos. Stimulation with FSH with low LH is now more common and has been proven to be an effective alternative to eCG protocols in terms of embryo quality [41-43]. However, in contrast to eCG, this type of FSH results in lower concentrations of estradiol in serum and follicular fluid, and of progesterone in serum [35,44]. When LH bioactivity is completely absent upon stimulation as with human recombinant FSH, development of preovulatory follicles still takes place but these follicles have a markedly reduced estradiol concentration and contain oocytes that lack

cytoplasmic maturation shortly before ovulation [45]. Therefore, balanced amounts of both FSH and LH are required for proper stimulation of follicles in the cow.

3. Discriminating competent follicles

The concept of developmental competence is not clearly defined since no particular mechanism is associated with it. But, it is believed that the acquisition of developmental competence is associated with different changes like, the synthesis and accumulation of specific RNAs and proteins, relocation of cytoplasmic organelles such as cortical granules, lipids and mitochondria. (See for review on intra-ovarian regulation [46]). The consequence of failing in any of these processes results in developmental failure. Assisted reproductive technologies routinely use controlled ovarian stimulation for oocyte recruitment and ovulation induction. Thus, an increased number of oocytes can be collected, though at the possible risk that not all gametes will show the same developmental competence [28,47]. This heterogeneity is probably due to intrinsic differences in the oocytes. It is well known that the hormonal milieu of the follicles is altered in cows stimulated with exogenous gonadotropin to a varying degree depending partially on the type of protocol and the hormonal treatment used [40,48-51]. We demonstrated that follicular concentrations of steroids are influenced by the size of the preovulatory follicle after oFSH stimulation [52]. However, as yet, the exact relationship between oocyte developmental competence and its respective intra-follicular environment is currently unknown. Nevertheless, in sheep, [53] and human [54], the physiological state of the follicles appears to affect subsequent oocyte maturation and competence in vivo. Asynchrony of follicle and oocyte maturation occurs after superovulation and may reduce oocyte developmental competence [28,47].

Although, we do not have proven reliable criteria to enable us to distinguish clearly between follicles with competent and non-competent oocytes, the steroid concentrations in the fluid of follicles can be used as indicator for competence. Oocytes collected from cows stimulated with recombinant human FSH have shown decreased concentrations of estradiol at onset of maturation, which were related to impaired distribution of cortical granules to the periphery at finishing of maturation. Normal distribution of these granules is considered a well known sign for developmental competence [45]. Steroid hormones are involved in a wide array of physiological responses, including regulation of glucose [55] and lipids, for instance, in aromatase-deficient (ArKO) mice, exogenous estradiol is necessary to maintain the gene expression and enzyme activity of the genes involved in hepatic lipid metabolism. Steroid hormones have been shown to regulate cell cycle progression [56,57], inhibition of apoptosis [58], and modulation of calcium release [59,60]. In the mammalian ovary, the follicle is the major site of synthesis and secretion of steroid hormones during preovulatory development and maturation of the oocyte. Regulation of steroid production by the ovarian follicular cells varies remarkably at different stages of development. During the preovulatory period, the selected dominant follicle is characterized by cyclical fluctuations in the levels of these hormones [61]. Before the preovulatory LH surge, granulosa cells synthesize and secrete estrogen, while after LH, granulosa cells luteinize and secrete more progesterone in concert with decreases in mRNA for 17alpha-hydoxylase and P450 aromatase [62]. The specificity of the steroid actions is due to the presence of intracellular receptor proteins. Despite the wealth of information about steroid receptors in different tissues and their importance in reproduction, only the receptor for estradiol ERβ mRNA has been identified in bovine oocyte [63]. Progesterone receptor mRNA in granulosa cells of the bovine preovulatory follicles is transiently induced within 5 to 7 h of the LH surge [64-66].

However, nothing is known about the expression of nuclear or membrane progesterone receptors in the oocyte of any mammalian species. In primates, androgen receptor (AR) mRNA activity is essential to early follicular development and oocyte quality [67], and in rats, complete disruption of AR activity is associated with intensive granulosa cell apoptosis in preovulatory follicles and poor quality cumulus oocyte complexes (COCs) [68]. Further, androgen receptors have been reported to translocate from the oocyte cytoplasm to GV, and then to the nucleolus suggesting a role as a ligand-activated, transcriptional factor [69]. In view of these observations, the identification and characterization of the patterns of mRNA changes, and functional analysis of the steroid hormone receptors that are expressed in the oocyte, if any, may provide a fundamental understanding of the critical roles of steroids during oocyte maturation in vivo. In clinical practice, there is a clear need to optimize the ovarian stimulation protocol, and proper design of superovulatory treatment should consider, LH concentration and half life in the FSH preparation, and steroid content and steroidogenic enzyme expression in the preovulatory follicles.

Therefore, it was assumed that functional preovulatory-sized follicles showing the changes in steroid concentrations as reported for untreated, normal cyclic cows primarily enclose competent oocytes.

4. Effects of superovulation on follicles and oocytes

Stimulation with gonadotropins not only affects the release patterns of endogenous hormones [30,31,70] but is also dependent of the developmental state of the follicles at onset of treatment. Since the cells of the follicular wall mediate the actions of the gonadotropin by an array of growth factors (For review see [71]) stimulating and inhibiting proliferation and differentiation of these cells, the effect of the gonadotropin on maturation of the oocyte varies markedly with size and state of atresia of the follicles (For review see [72]). Accordingly, the competence of oocytes to develop in vitro into blastocysts is related to the origin of the oocyte [73,74]. For example, oocytes collected from follicles in the presence of a growing dominant follicle (DF) show a reduced competence compared to oocytes recovered in the absence of a DF [75,76]. In practice, absence of the DF at onset of superovulation has been reported to increase the response [77-79] although removal of the DF by puncture at 38 to 46 h before stimulation did increase the number of viable embryos only in cows but not in heifers compared to animals that were not punctured [74]. Whether the DF exerts its effect on the remainder of the follicles > 2 mm by intra-ovarian or endocrine routes is not solved. In cows repeatedly treated with eCG/anti-eCG for 2 years the proportion of cows not showing a preovulatory LH surge was substantially reduced when the DF was removed (unpublished, PLAM Vos, B Aguilar, SJ Dieleman). Moreover, follicles < 4 mm may survive and participate in the next follicular wave [80].

Selection of animals showing a regular estrous cycle and timing of gonadotropin administration in relation to the follicular wave is prerequisite investigating biological mechanisms that determine the competence of the oocyte.

4.1. Superovulation with eCG/anti-eCG

In our early experiments, cows were administered 3,000 IU eCG (Folligon; Intervet International BV, Boxmeer, The Netherlands; heifers 2,500 IU) at Day 10 of a pre-

synchronized cycle and prostaglandin (PG) 48 h later. On average the endogenous LH surge occurred at 44 h (range 30 to 52 h) after PG, and anti-eCG (Neutra-PMSG; Intervet International BV) was administered i.v. at 6 h after the maximum of the LH surge in a dose sufficient to neutralize 3,000 IU eCG within 1 h [24]. In later experiments, norgestomet (Crestar; Intervet International) was implanted simultaneously with the administration of eCG [81] prolonging the period of stimulated follicular development by suppression of the LH surge. Subsequent administration of GnRH at 54 h after PG induced an LH surge at a controlled time which facilitates administration of anti-eCG and collection of oocytes and embryos at precisely defined stages of development.

The majority of the stimulated populations of follicles per cow showed a mixture of follicles with steroid concentrations in the fluid conform to or deviating from those reported for the preovulatory follicle of untreated cows during final maturation [61,82,83]. In cows treated with saline in stead of anti-eCG, significantly higher numbers of follicles were found with deviating, high estradiol concentrations in the fluid shortly before ovulation when the oocyte should have completed maturation. Neutralization of the eCG apparently did not affect the concentration of progesterone [83].

Oocytes from stimulated follicles at ovulation from cows with or without controlled LH surge only rarely lacked an expanded cumulus (3.5%). They also had a 2-fold higher competence to develop to the blastocyst stage after further in-vitro fertilization and co-culture compared to oocytes derived from 2 to 5 mm slaughterhouse follicles [81,84]. This difference may be due to the difference in origin of the oocytes: in vivo matured vs. immature. When in-vivo prematured oocytes were used for in-vitro maturation in stead of immature oocytes the blastocyst formation rate still remained significantly less than from in-vivo matured oocytes (26 vs. 41%, respectively; [85]). Although this finding strongly indicates that in-vivo maturation enhances competence, replacement in later experiments of the porcine FSH with rec hFSH in the maturation medium invalidated the hypothesis (unpublished, MM Bevers, SJ Dieleman).

Prolongation of the period of stimulated follicular development in the norgestomet/GnRH-controlled LH cows increased the ovulation rate but not the number of viable embryos [81]. Since a marked decrease or even absence of secretion activity was observed in the epithelium of the ampulla close to the junction with the isthmus, it was suggested that the milieu for early embryonic development was not optimum [81]. An explanation for this phenomenon could be the delayed switch from progesterone to estradiol dominance over the oviduct due to the norgestomet treatment. Products of the epithelium play a role in early embryonic development and their secretion is controlled by steroid hormone [86].

4.2. Superovulation using FSH with controlled LH surge

From 1999 onwards our research was continued using ovine FSH (Ovagen ICP, Auckland, New Zealand) as gonadotropin with a norgestomet/GnRH-controlled LH surge (Fig. 1). Only occasionally (13/185 animals) complete suppression of the release of the endogenous LH surge failed when the LH concentration in the peripheral blood started to increase 10 h before termination of suppression and administration of GnRH, and rarely (2/185) an LH surge occurred during suppression [87].

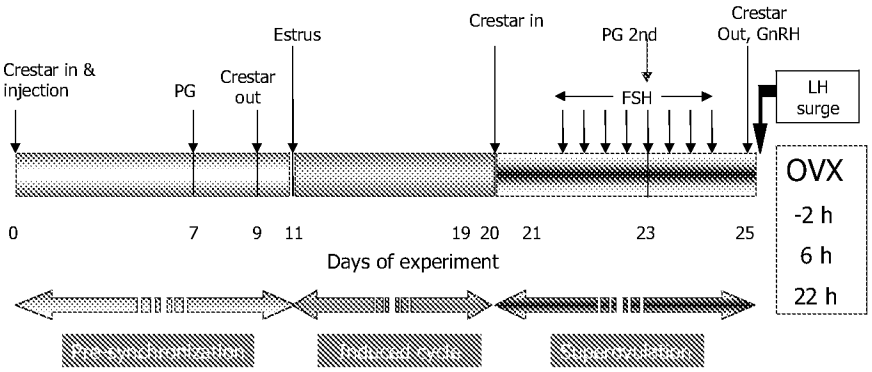

Figure 1. Schedule of treatment for pre-synchronization and superovulation with a Crestar/GnRH-controlled LH surge to obtain oocytes at specific times of development; PG=prostaglandin. The precise timing of the administration of PG during the FSH treatment was determined at intervals of 1 h to allow for periods of 1 h between each cow at ovariectomy, which is the time needed to collect all follicles. Similarly, removal of the 2nd earimplant (Crestar out) and administration of GnRH were carried out at 1 h intervals; a maximum of 4 cows was used every treatment run. (Adapted from [52]).

The number of stimulated follicles per cow varied substantially, e.g. 23.9 ± 12.1 SD (n=50 cows) upon ovum pick-up (OPU) shortly before or at 22 h after the induced LH surge [88]. A typical distribution per size category during final maturation is presented in Table 1 [89]. As with eCG/anti-eCG stimulation the oFSH protocol produces a mixed population of follicles with normal and deviating steroid profiles. Criteria to select follicles with presumably competent oocytes were derived comparing the follicular fluid concentrations in preovulatory-sized follicles with those of 5 to 8 mm follicles (Fig. 2; [52]) resulting in about 60% of the follicles > 10 mm with a normal steroid profile. However, in this normal category, the estradiol concentration before the LH surge was about half of the corresponding concentration in preovulatory follicles from non-treated normally cyclic cows. The lower estradiol concentration coincided with lower concentrations of its precursor androstenedione that is synthesized in the theca cell layer. It was suggested that this incident might be due to the low LH bioactivity of the oFSH preparation used. A major finding is the significant increase with size of the concentration of progesterone in particular shortly before ovulation when luteinization of the follicular wall should be completed. In Table 2 steroid concentrations are presented for normal and deviant follicles from which oocytes have been used to investigate the transcriptome [89].

Oocytes from non-selected stimulated follicles showed a marked competence for further early embryonic development in vitro (Fig. 3; [88]). Apparently, there was no difference between in-vitro and in-vivo routes for maturation with regards to the proportions of developed embryos which might be explained by the use of rec hFSH in the maturation medium as discussed above. However, significant improvement of traits was observed following in-vivo maturation such as numerical chromosome abnormality [88]. The degree of mixoploidy increases from Day 2 to 5 after insemination in embryos flushed from cows

stimulated with the oFSH protocol with controlled LH surge but levels out much earlier than in entirely in-vitro produced embryos [90].

High but variable numbers of embryos can be collected at specific stages of early development (11 per cow, n=99 cows; [87]) with high proportions characterized as viable (78% at the 8-cell stage and >50% at later stages after MET).

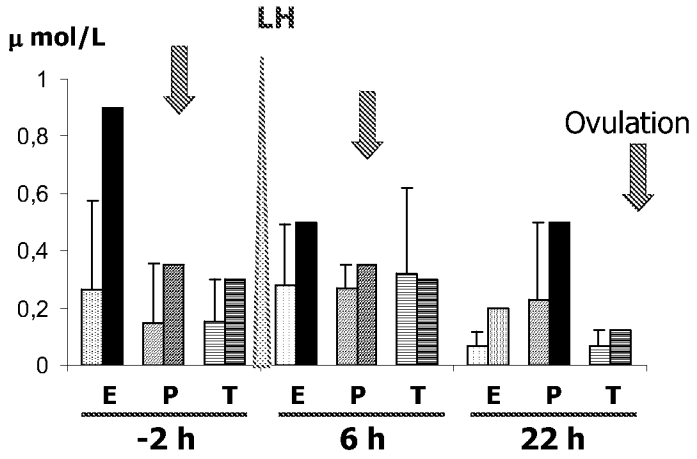

Figure 2. Threshold levels of steroid concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; E: estradiol, P: progesterone, T: testosterone. Of each pair of bars, the left bars represent the mean ± SD steroid concentration for follicles 5 to 8 mm, and the right bars are the threshold value for large preovulatory follicles with in black the predominant steroid value.

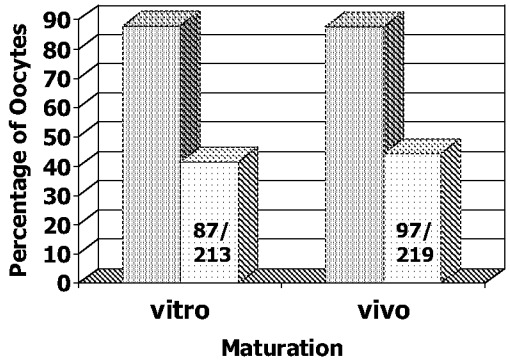

Figure 3. Competence of oocytes after in-vitro vs. in-vivo maturation. Oocytes were collected by OPU (n=4 experiments) from FSH-treated cows (n=36) with controlled LH surge at 2 h before and at 22 h after the induced LH surge, and matured in vitro or directly fertilized preceding further culture, respectively. Left bars: cleavage, right bars formation of morulae and blastocysts; numbers indicate number of embryos/oocytes collected. (Adapted from [88]).

4.3. Conclusions

Superovulation using norgestomet/GnRH to control the time of the LH surge facilitates collection of oocytes and embryos at defined stages of development although also non-competent and non-viable cells constitute part, requiring selection. In addition granulosa and cumulus cells can be recovered of interest investigating regulation and signaling between somatic cells and gamete or deriving non-invasive markers to establish competence and viability in IVF programs. Although regular numbers are obtained it is not yet clear whether the prolongation of stimulated follicular development would lead to higher yields of embryos in practice. Apparently the norgestomet/GnRH treatment does not affect in-vitro competence of oocytes but it remains to be resolved to what degree the oviductal milieu has become detrimental for early embryonic development.

The stimulation with controlled LH surge can be carried out with either eCG/anti-eCG or with FSH preparations and can be applied investigating biological processes in vivo such as apoptosis in embryos [91,92] and expression of genes [89,93]. At the molecular level differences in effect on reproductive cells can be foreseen between gonadotropin preparations due to mode and degree of glycosylation of the protein and to LH bioactivity.

5. Superovulation as a tool to study transcriptomics of competent oocytes

Follicle development was stimulated in Holstein-Friesian cows (n=40) using our standard protocol [93] with oFSH Ovagen ICP, Auckland, New Zealand) and a Crestar/GnRH-controlled LH surge (Intervet International B.V., Boxmeer, The Netherlands). Cows were allocated at random to three experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and completion (22 h after LH) of maturation to determine changes in mRNA expression related to resumption of meiosis in vivo (Fig. 1). Most of this study into the transcriptomics of the oocyte was done in the framework of the PhD-thesis of O.A. Algriany between 2003 and 2007 [89].

The growing mammalian oocyte, although morphologically simple, undergoes a series of discrete differentiation events. A relatively large number of genes are required to program its entire development. A small fraction of these genes are oocyte specific [94] while the large majority are expressed both in the oocyte and in somatic cells. Transcription and repression of genes is a dynamic process that can be expected to vary in the oocyte with the culture conditions. In order to assess which genes may be regulated by specific stimuli, it is necessary to have the capability of examining genes under a variety of exposure conditions. The triggers for change in gene expression in oocytes are critical for understanding the molecular mechanism of oocyte maturation. In fact, the information that is currently available on molecular mechanisms regulating oocyte maturation has been largely obtained from studies using in-vitro matured oocytes. Perhaps even more important, in the cow, most in-vitro studies have been carried out with oocytes collected from small or medium sized follicles (3 to 6 mm follicles), which lack the prematuration stage [73,88]. Prematuration begins at an average follicle diameter of 8.5 mm, that is the beginning of the difference in growth between the two largest follicles [95] and is associated with a differentiation of the concentration of estradiol [96].

Because in vivo oocyte maturation relies on a subtle balance between different follicular regulatory compounds, and also probably between different oocyte receptors, the molecular and biochemical alterations triggered by artificial ligands in vitro may not

necessarily reflect the normal in-vivo processes. The complexity of meiotic resumption regulatory mechanisms is also well demonstrated in bovine oocytes [97]. The bovine preovulatory follicle appears an attractive experimental model for study of the regulation of oocyte maturation and its ability to develop after fertilization. The preovulatory follicle contains sufficient follicular fluid for the analysis of steroid, proteins and various regulatory compounds. It contains also sufficient amounts of granulosa and cumulus cells, which offers an excellent opportunity to investigate functional interactions between various regulatory factors.

Differentially expressed genes between the oocytes exposed to LH and those collected before can help us understand the molecular basis of meiotic resumption in vivo. The identification and characterization of oocyte genes expressed exclusively or preferentially in the 6 h in-vivo matured oocyte will hopefully shed light on the mechanisms of the maturation process and provide useful information for the development of efficient maturation media. The suppression subtractive hybridization (SSH) method allows identifying overexpressed genes (designated forward +SSH) but also underexpressed genes (designated reverse -SSH) by exchanging the driver and tester populations during the procedure (Clontech, Palo Alto, CA, U.S.A.) [98-103]. SSH is a still widely used technique since it enables the recovery of abundant as well as low copy number mRNA transcripts. However, because it still needs a lot of initiating RNA which will burden using in-vivo matured oocytes, the use of the *S*witch *M*echanism *A*t the 5` end of the *R*everse *T*ranscript (SMART) amplification method overcomes this limitation.

The development of microarray technologies permits thousand of genes to be screened in a single experiment to establish differential gene expression in treated versus control cells and population. Consequently, the use of DNA microarray should significantly aid in minimizing the effort required to screen the many variables required to effectively examine gene expression patterns. Microarrays are developed to represent expressed mRNA transcripts (cDNA arrays), or distinguishable portion of an mRNA transcript (oligonucleotide arrays). The popularity in use of this technique is demonstrated by the exponential growth in publication using microarrays since its inception in 1995. Microarrays have been widely used to study issues in pathology, pharmacology, oncology, cell biology and recently, oocytes [94,104,105].

Different techniques have been used for gene discovery to design oocyte specific cDNA microarrays for possible use in assessing reproductive technologies performance. Two of these techniques employ a method for selectively segregating cDNA clones or fragments found in one cell or tissue population and absent in another. At present, the complete gene database for bovine is becoming available. Therefore, genomic information must be employed to construct a microarray to use in screening transcripts in the bovine oocyte. The ideal approach to gene expression profiling is to use full genome microarrays to identify genes up or down-regulated in response to certain treatment. However, because microarrays are not likely to become a routine test in the near future, reproductive biotechnology studies will probably require identifying a small subset of genes whose expression can be applied in the development of gene-based quality test. Therefore, we used SSH and microarray technique and oocytes matured in vivo to identify genes involved in regulating the maturation of bovine oocyte, suggesting that LH and maturation in vivo is instrumental in regulating several aspects of oocyte function.

5.1. Collection and processing of oocytes

Presumptive competent oocytes were selected on the basis of the steroid profile in the enclosing follicle. Pools of 10 to 14 denuded oocytes were assigned to replicates for Suppression Subtractive Hybridization (SSH) and validation by QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. For some studies QPCR analysis was also performed with "non-competent" in-vivo FSH-stimulated oocytes, in-vitro matured oocytes from slaughterhouse ovaries, and in-vitro [106] and in-vivo [107] produced expanded blastocysts. Total RNA was isolated using a microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit; Stratagen, San Diego, CA, U.S.A.). The SMART^{TM} PCR cDNA Synthesis Kit (Clontech) was used to maximize cDNA yields prior to the subtraction. The PCR-Select cDNA Subtraction Kit (Clontech) was used for SSH to isolate and enrich for gene sequences differentially-expressed between the two pools of oocytes: 1) collected before LH surge (-2 h, n=30) as driver and 2) exposed to LH (6 h, n=30) as tester. The subtracted material was then cloned as described by Algriany et al. [108]. Microarray preparation, hybridization and analysis as well as QPCR (Bio-Rad Laboratories, Hercules, CA, U.S.A.) were carried according to Sirard et al. [105,109]; for primers used see [108,110,111].

5.2. Differential expression at resumption of meiosis

Using SSH and defining a 1.3 fold difference as threshold, 115 regulated genes were identified from initially 945 DNA clones between the –2 h and 6 h after LH oocytes. This relatively small difference in the gene expression pattern due to LH surge may point to the fact that only a small subset of genes needed to regulate the meiotic resumption and developmental competence. Microarray analysis has uncovered novel mRNAs with potential roles in proper oocyte function, maturation and/or meiotic competence. We identified important changes in genes involved in cell cycle regulation, signal transduction, transcription and mRNA processing, cytoskeleton, cell adhesion, as well as in metabolism [108]. Real time QPCR analysis showed a significant 4- to 5-fold up-regulation for some genes: cAMP phosphodiesterase and Cytochrome c oxidase subunit VIII (Fig. 4). A tendency of approx. 3-fold up-regulation for: e.g. G-protein γ 12, metabotropic Glutamate receptor 5, PPAR binding protein, while the expression of some other genes was not different between "competent" oocytes before vs. after onset of resumption of meiosis [108].

Following the identification of these genes at the mRNA level, the challenge is to utilize efficiently this information to develop a better understanding of meiotic resumption mechanism. The proteomic approach may provide information that could not be obtained at the RNA level, due either to poor correlation between mRNA and protein levels or due to post-translational modifications that may result in several isoforms generated from one mRNA. For many genes identified, both the putative ligands that activate them and their targets of action remain unknown and represent challenges for future studies to unravel the mechanism of oocyte maturation and developing efficient IVM system.

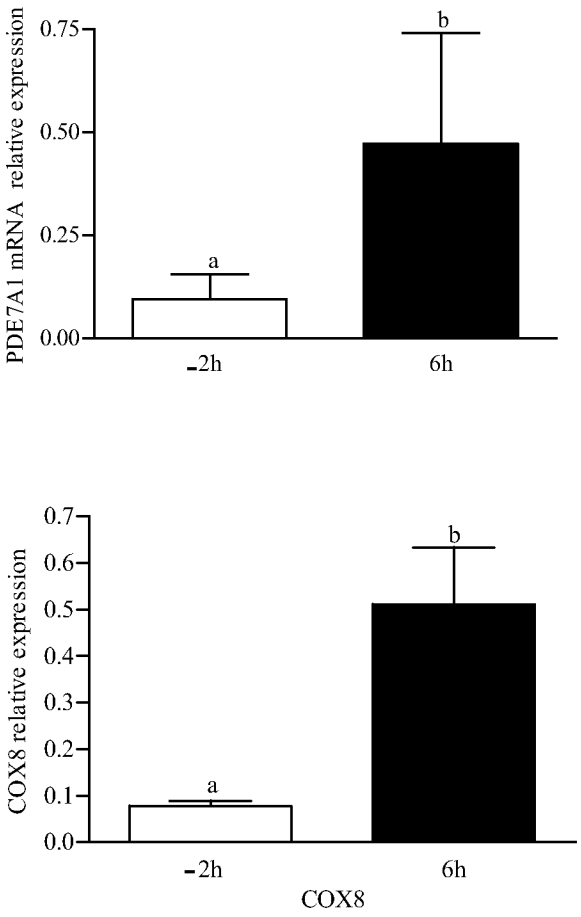

Figure 4. Real-time PCR analysis of mRNA expression of 2 of 10 genes selected from microarray results; data are shown as mean ± SEM of the mRNA level of the oocytes collected 2 h pre LH surge and the oocytes collected 6 h following the LH surge. Upper and lower panels: cAMP phosphodiesterase and Cytochrome c oxidase subunit VIII expression, respectively. Different letters indicate statistical significance between the two groups as determined by unpaired Student's *t*-test; $P<0.05$ was considered statistically significant. (Adapted from [108]).

5.3. Molecular motors and chromosome segregation

While the spindle and chromosome morphology of the meiotic events is known for over a century, most of the basic molecular mechanisms regulating the chromosome segregation in mammalian oocytes are widely unknown. Meiosis within the oocyte must be precisely regulated to ensure proper division of the genetic material. Missegregation of chromosomes results in aneuploidy and could lead to inviability. An estimated 10 to 30% of fertilized human eggs have the wrong number of chromosomes, with most of these being either trisomic or monosomic. This has profound clinical consequences: approximately one-third of all miscarriages are aneuploid, which makes it the leading known cause of pregnancy

loss and, among conceptions that survive to term, aneuploidy is the main genetic cause of developmental disabilities and mental retardation [112].

The real-time QPCR analysis of six of the genes having a significant role in the spindle formation and maintenance of accurate chromosomal segregation and construction of the cytoskeleton showed deregulated and aberrant mRNAs levels in, oocytes from follicles with deviant steroid profiles compared to normal [110]. In particular, the expression levels at onset of final maturation that is at 2 h before LH were significantly 5- to 10-fold lower in oocytes from follicles with deviant steroid profiles. After resumption of meiosis these differences were reduced to absent. In in-vitro cultured oocytes at corresponding times of maturation the expression levels were in general in between the levels of the in-vivo normal and deviant oocytes; the genes examined were KIF3A, Cytoplasmic dynein, Myosin regulatory light chain, Formin 2 like, Par3 and Aurora-A. Only Par3 and Myosin regulatory light chain were evidently expressed in expanded blastocysts, and were lower in in-vitro produced embryos than in the embryos obtained after flushing the superovulated cows [110]. This may explain the chromosomal abnormality frequently seen in the oocyte and early human preimplantation embryos cultured in vitro, which is commonly associated with impaired cleavage, poor embryo quality and increased fragmentation, all of which may compromise the implantation potential of the embryos [113-115].

While the polarity in mammalian embryos is a well-known phenomenon, existing polarity in mammalian oocyte is still controversial [116-118] and little is known about the genes regulating polarity and related activity in oocytes. We identified several transcripts in the oocyte known to play a role in polarity axis formation like par-3, formin, KIF3, β-catenin and CDC42 (unpublished, Algriany et al.). Organelles and cortical actin distribute asymmetrically in the oocyte of many species as the dorsal/ ventral axis forms [118]. The identification of genes regulating polarity in the oocyte and the recent finding that Par-3 protein is associated with meiotic spindles [119], may point to their important function during meiotic resumption and possible role in oocyte polarity.

Moreover, although most of the genes identified [110] are also common to somatic cells during mitosis, there is a fundamental difference. The chromatids are held together during prometaphase of meiosis II only at the centromere, whereas during prometaphase of mitosis they are joined (at least initially) along their entire length. This raises the interesting possibility that chromosome disjunction during mitosis also requires two different sets of machinery. One that is normally present during meiosis I that separates the chromatid arms, and another normally found in meiosis II (or during the preceding interphase) that leads to separation in the centromere region. In the future, it will be important to determine the differences between the two mechanisms that operate during meiosis to separate the chromosomes. The separation of sister chromatids is a complex process and there are certainly other factors involved in regulating the attachments and separation of sister chromatids.

5.4. Genes involved in lipid metabolism

Because the action of the products of the different transcripts identified in relation to resumption of meiosis, molecular motors and chromosome segregation, is ATP-dependent, and because the correlation of oocyte ATP content and developmental competence is well established, energy requirements constitute an important factor to accomplish competence

during maturation. In general, lipids form an energy source but there is a lack of information on the role of lipids as energy source in bovine. Therefore, based on information in somatic cells, we investigated the involvement of various pathways for lipid transport, β-oxidation and *de-novo* fatty acids synthesis during final maturation of bovine oocyte using QPCR.

A full understanding of the physiological effect of maturation in vivo on gene expression requires identification of the transcripts having an impact on metabolic pathways, their mode of action, and their consequences for growth, differentiation and survival. At a more practical level, gene identification is essential for formulating a successful maturation medium to support oocyte development after fertilization.

Changes associated with ultrastructure of the growing oocytes related to the accumulation of nutrients like lipids are prerequisite of energy for meiotic resumption and subsequent embryonic development. The origin of lipids reaching the oocyte is not fully understood. Lipids stored in the oocyte have been shown to be accumulated in the oocyte during follicular development [120] and start to decrease during the maturation process [121]. Kim et al. [122] showed that lipid content in bovine oocytes reflects the lipid content in the maturation medium, indicating that lipids accumulating in the oocytes must originate from the medium. It is not known whether these lipids pass via the junctions between the oocyte and its surrounding cumulus cells or are taken up directly from the follicular fluid. Since many genes are conserved across human and animal species, function of certain genes can be extrapolated. Therefore, mRNAs representing the major metabolic pathways involved in lipid metabolism were investigated in normal oocytes [111]. From the results, it is possible to suggest a model for long-chain fatty acid (LCFA) transport into the oocyte. The fatty acids are translocated from the extracellular environment to the cytoplasm by the fatty acids translocase (FAT/CD36) and then solubilized and transported by fatty acid binding proteins (FABPs) to the site where they are metabolized [123,124]. Once transported across the membrane, LCFA are targeted to specific metabolic fates. These findings together with the higher level of CPT-1 mRNA propose that fatty acid is directly required for meiotic resumption. Further, they indicate that β-oxidation is the major pathway contributing to the energy requirement during oocyte maturation and increased rate of lipogenesis at the blastocyst stage which may be needed to support earlier embryogenesis. It was clear that a switch from import of lipids to synthesis occurred between oocyte and blastocyst stage (Fig. 5) [125]. Then, the mRNA involved in lipid metabolism were compared to deviant oocytes collected from stimulated cows and those matured in vitro to pinpoint impairment of particular pathways of lipid metabolism. The aberrant levels of several mRNAs may indicate that intracellular fatty acid composition is not proper, decreased β-oxidation and may explain the lower progression of meiosis, lower ATP levels and lower developmental competence of these oocytes. The significant lower mRNA levels of Acetyl CoA carboxylase α (ACCα) the main enzyme controlling *de-novo* fatty acid synthesis may explain partially the lower developmental competence of in-vitro produced blastocysts. This may indicate a change in the quantity of synthesized lipid, resulting in a lack of certain LCFAs needed for membrane integrity and structure.

Although our study measured mRNA of the main pathway of lipid metabolism, the interpretation of the data on fatty acid metabolism has a number of limitations. For instance, considering that over 30 reactions are required to convert acetyl-CoA to triglycerides, there could be many steps or genes that control the yield of end product. Beside that, in addition to

fatty acids (FA), glucose is another main oxidized metabolic substrate, however, its role during oocyte maturation as energy source is controversial [126-128]. Interactions between

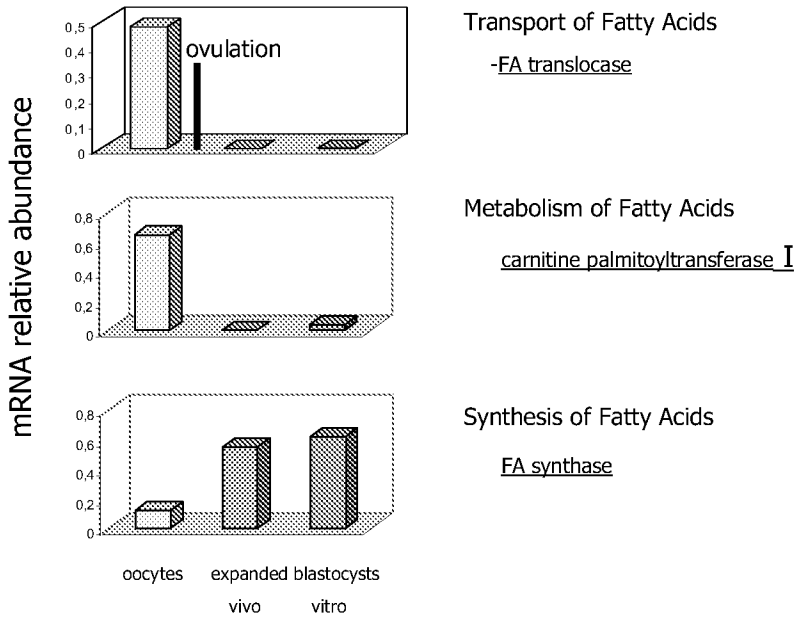

Figure 5. Switch from import of lipids into the oocyte (left bars) to synthesis of lipids in expanded blastocysts obtained in vivo after eCG superovulation (middle bars) and in vitro from slaughterhouse oocytes (right bars). (Adapted from [125]).

these substrates are thought to control the extent of their respective oxidation, i.e., to control the reciprocal relation between glucose and FA oxidation. However, which of the two substrates, glucose or FA, is the primary regulator of energy in the oocyte is not clearly known and needs further investigations.

5.5. Conclusions

Using oocytes from FSH-stimulated cows with a controlled LH surge has revealed many new basic properties of the bovine oocyte in relation to competence. We now know that mechanisms of meiotic arrest and resumption require different phosphodiesterases (PDE7), the involvement of G-proteins, and regulators of G-protein signaling (RGS) in meiotic resumption, molecular components involved in chromatids separation, regulation of Ca^{2+} oscillatory activity and cell cycle regulation. Most of these genes were identified for the first time in the mammalian oocyte. However, the challenge is to identify the potential ligands that activate these genes, which may provide an answer for how meiotic arrest is released. Future research will certainly provide answers to the open questions regarding these issues. In addition, the FSH-stimulated oocytes have provided crucial information regarding mRNAs involved in correct translocation of organelles and segregation of chromosomes possibly explaining disturbed polar body emission and defects in cytoplasmic maturation as commonly observed in deviant oocytes from stimulated animals and in in-vitro matured oocytes. Finally, new information has been obtained regarding long chain fatty acid transport into the oocytes and regulation of energy requirement during maturation and blastocyst stage

using lipid as a substrate, which may be used to adapt in-vitro culture of oocytes in many applications.

6. Perspectives

Although knowledge on oocyte maturation has grown rapidly during the last few years, the field needs to include completion of the molecular details, determination of key molecular structures, assignment of physiological functions, elucidation of physiological regulatory mechanisms, and exploration of interfaces with other cellular systems. As a result of our studies some routes for further investigation can be identified. First, we need proof that compounds such as steroids in the follicular fluid can be used as marker for oocyte competence for example by culturing single oocytes and determining rearrangement of organelles and competence to develop into viable blastocysts. Secondly, hybridization in microarrays of FSH-stimulated oocytes against oocytes derived from other routes such as in vitro, cross species [129] and last-but-not-least the "golden" standard from untreated animals might reveal eventual negative effects of the gonadotropin used. In this respect the cow can be used as model to improve IVF procedures in man. Moreover, comparing the expression of genes and using proteomics in the surrounding somatic granulosa and cumulus cells may lead to non-invasive clinical applications in human IVF.

Acknowledgements

It is impossible to be complete but here are some of the people I am most indebted to who have helped me with dedication round-the-clock which ultimately resulted in achieving the 2007 A.E.T.E. Pioneer award. I gladly accept the award on behalf of all of us. First of all the two colleagues who were always encouraging and interested to participate, the composed Mart Bevers with his great talent for in-vitro culture and Theo Kruip rich in ideas and always ready for a jest. Unfortunately, they both are not alive anymore to share this happening. We worked together for decades; that is anchored in memory! Next I should mention my much valued colleague and friend Peter Vos for his marvelous support and being a terrific sparring partner in science, and the post-docs Begoña Aguilar, Peter Hendriksen and Mitsuhiro Takagi for their specific insights in areas that were not directly my expertise. The team at the laboratory was without any doubt most essential to organize experiments and to get all analyses precisely done and in time!, in particular Thea Blankenstein, Christine Oei, Ad van de Poll, Leni van Tol and Elly Zeinstra. During their PhD study Ellen van de Leemput, Hiemke Knijn, and Omran Algriany, and many other PhD-students and guests from abroad were invaluable helping with collection of oocytes and embryos and with analysis; the many veterinary and biology and agricultural sciences Master students of whom Jan Joop Harkema, Bram van Schaik, Wilma Fokker, Elske de Boer, Henry Seinen, Sander van Gastel, Huijbert Groenendaal, Elsa van Rhenen and Annemarie Waijer are acknowledged. Last but not least, the animal caretakers and their supervisor Hans Lutz, and the surgeons Bert van der Weyden, Herman Jonkers, Maarten Pieterse and the surgery assistants are recognized.

Evidently this 'opus' could not have been accomplished without the generous collaboration of many people in other laboratories. I express my sincere appreciation to all of whom in particular Poul Maddox-Hyttel (Copenhagen, Denmark), Heiner Niemann and Christine Wrenzycki (Mariensee, Germany) and Marc-André Sirard (Quebec, Canada) and their colleagues. The generous supply of in-vitro embryos by Sybrand Merton (Holland Genetics, Harfsen, The Netherlands) and the donation of hormones by Intervet International

B.V. (Boxmeer, The Netherlands) is greatly appreciated. Finally, I heartily acknowledge the ever inspiring atmosphere and the tremendous collegiality I have experienced year upon year at the Annual Meetings of the A.E.T.E. and the I.E.T.S. It has been like a family.

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Journal of Reproduction and Development, Vol. 40, No. 3, 1994

—Technical Note—

Superovulation Using CIDR® in Holstein Cows

Yasuaki IWAZUMI, Yutaka FUKUI[1)], Rodolfo Bitarra VARGAS[1)], Chiyo NAKANO, Noriyuki SATO[2)], Makoto FURUDATE[2)], Kazue OHSAKI[3)] and Shigenori MATSUZAKI

Hokkaido Livestock Improvement Association, Komasato, Kunneppu-cho, Tokoro-gun, 099–14, [1)] Laboratory of Animal Genetics and Reproduction, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, [2)] Hokuren Livestock Experiment and Training Farm, Komasato, Kunneppu-cho, Tokoro-gun 099–14, and [3)] Laboratory of Theriogenology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan

Abstract. Three trials were conducted to investigate the superovulatory response of Control Internal Drug Releasing device (CIDR®). Forty-six Holstein cows were divided into three treatment groups per trial; CIDR® without progesterone (P_4) (Control), CIDR® containing 1.9 g P_4 for 12 days (CIDR®-1) and the two CIDRs® with P_4 replacing with a new CIDR® on day 9 of the 12 day insertion period (CIDR®-2). Follicle stimulating hormone (FSH) were administered on day 9 up to the time of CIDR® removal and additional prostaglandin $F_2\alpha$ injections were done on day 11 of the 12 day insertion period. Ovulation rate, recovered and transferable embryos, and P_4 and estradiol-17β (E_2) levels in blood plasma were evaluated and compared among the treatment groups.

There were no statistical differences on the mean (± SEM) ovulation rate, recovered and transferable embryos. An encouraging results of transferable embryos were obtained in the 3 treatment groups (80.7%, 69.8%, and 61.4% for Control, CIDR®-1 and CIDR®-2 respectively). No existing evidence of correlation between plasma P_4 and ovulation rate at the time of AI (day 13) was observed. Plasma P_4 and E_2 concentrations were characterized by the physiological variability of individual cow and heterogeneous follicular development following superovulatory treatments. The results suggest that the use of CIDR® presents a satisfactory superovulatory regimen which could prove applicable for embryo production under field conditions. However, further research is necessary to elucidate factors involved in the improvements of the regimen.

Key words: CIDR®, Cows, Superovulation, FSH and $PGF_2\alpha$.

(J. Reprod. Dev. 40: 259–266, 1994)

Superovulation is a key element of embryo transfer (ET) program [1] to increase the supply of embryos from animals of superior genetic quality [2]. Application of ET requires an acceptable developed superovulatory procedures for planned cattle breeding [3]. Armstrong [1] emphasized that superovulation is determined by different interacting factors such as those influencing ovulatory response of donors, fertilization and embryo viability.

In recent years, various routine methods were reported and initiated for superovulation and production of fertilized ova. These reports dealt with the mode and applicable uses of hormonal preparations like follicle stimulating hormone (FSH) [3–

Accepted for Publication: March 3, 1994
Correspondence to: Y. Fukui

12] and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) [4, 10, 13–15, 17]. Superovulation utilizes FSH to increase the number of viable ovulatory follicles and induces a rapid, synchronous return to estrus following $PGF_2\alpha$ treatment [4]. Accordingly, $PGF_2\alpha$ or its analogues has been the greatest advance in superovulation methodology, because the optimal treatment can be initiated anytime between day 8 to 12 of the estrous cycle which results in the production of excellent embryos [2]. Also, superovulation with the application of intravaginal devices such as the control internal drug releasing device (CIDR™) and the progesterone releasing intravaginal device (PRID) with hormones (FSH plus $PGF_2\alpha$) were found to be of encouraging results in the previous studies [10, 11, 14, 18, 19].

Therefore, the present field trials were conducted at the first time in Japan to examine the superovulatory response of Holstein cows to CIDR™ combined with hormonal injections of FSH and $PGF_2\alpha$. The purpose of the present study were 1) to investigate a possibility of superovulation with CIDR™ in Holstein cows kept in Japan and 2) to compare the ovarian responses and embryo recovery in superovulated cows treated with placebo (CIDR™ not containing progesterone) and with one or two CIDR® treatment.

Materials and Methods

Forty-six Holstein cows, 3 to 9 years of age were used and designated in 3 trials with 3 treatment groups each, during the autumn (Trial A: 17 heads, October to November 1991), spring (Trial B: 14 heads, May to June 1992) and, again autumn (Trial C: 15 heads, October to November 1992) at Kitami Center, Hokkaido Livestock Improvement Association, Kunneppu-cho, Tokoro-gun, Hokkaido, Japan.

The CIDR® (Eazibreed Type B: The Carter Holt Harvey Plastic Products Ltd., New Zealand) is a tubular T-shaped device and comprises a moulded inner nylon spine and an outer coating of silicone elastometer. A CIDR® device was inserted into the vagina of the cows to enhance superovulation for 12 day insertion period (Insertion day: day 0). Cows were allotted to 3 treatment groups per trial; CIDR® without progesterone (P_4) (placebo: control) for 12 days, CIDR® containing 1.9 g P_4 for 12 days (CIDR®-1), and two CIDRs® with P_4 replacing by a new CIDR® on day 9 of the 12 day insertion period (CIDR®-2). Cows for the control groups were inserted CIDR® without P_4 (placebo) on day 2–4 of the estrous cycle while cows of the CIDR®-1 and CIDR®-2 were inserted CIDR® with P_4 at the unknown stages of the estrous cycle. Superovulation was induced with eight times intramuscular (IM) injections of a total of 44 mg FSH (Antorin: Denka Chemical Co., Japan) twice a day with approximately 12 h intervals in a declining dose (7, 7; 6, 6; 5, 5; 4, 4 mg; a.m. and p.m.) from day 9 to day 12 of the insertion period. Two IM injections of 20 mg and 15 mg prostaglandin $F_2\alpha$ ($PGF_2\alpha$ pronalgon-F; The Up-john Co., Japan), were administered in the morning and afternoon on day 11 of the insertion period, respectively. After the CIDR® withdrawal, estrus was observed and artificial insemination (AI) with 0.5 ml of frozen-thawed semen was performed on day 13 by the routine schedule at the station. Embryos were collected non-surgically on day 20 i.e., 7 days after the onset of estrus as described by Munro [11] and Hafez [2], and selected for direct transfer or cryopreservation on the basis of morphology [2, 3, 6, 7]. Rectal palpation [6] and an ultra sound scanning device were applied after embryo recovery to estimate the number of corpus luteum (number of ovulation) and follicles in the ovaries.

As shown in Fig. 1, blood was collected from the caudal vein by a heparinized vacutainers on day 0, 2, 9, 11, 12, 13 and 20 per animal in all the treatment groups. Immediately after blood collection plasma was separated by centrifugation at 1,600 g for 10 min and stored at −20 C until assay. P_4 and estradiol-17 (E_2) in plasma were measured by radioimmunoassay (RIA) as described by Aoyagi [10] and Mochizuki *et al.* [20]. The average recovery rate was 80.0% and the intra and inter assay coefficient of variations of P_4 and E_2 were 4.6–12% and

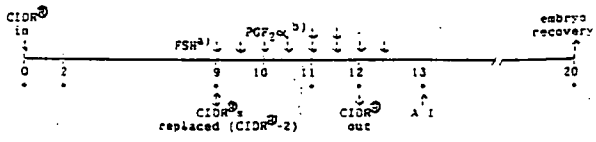

Fig. 1. Schedule of the treatments of the study. Figures below the line show the days after treatment (CIDR® insertion: day 0). *indicates the days of blood collections. a) Start of FSH injection on day 9 with a declining dose (7, 7; 6, 6; 5, 5; 4, 4 = 44 mg). b) Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) injection (20 and 15 mg = 35 mg) on day 11 of the 12 day insertion period.

6.2–16.0%, and 14.0–23.1% and 10.1–16.7%, respectively.

Ovulation rate, recovered and transferable embryos, and plasma P_4 and E_2 concentrations were analyzed using the general linear models procedure ($p<0.01$).

Results

Mean (± SEM) ovulation rate, recovered and transferable embryos of superovulated cows per treatment group/trial are presented in Tables 1 and 2, respectively. No significant difference was observed on the mean ovulation rate, number of recovered ova/embryos per cow and transferable embryos per recovered ova/embryos in all the treatment groups of the three trials (seasons). However, a high total percentage of transferable embryos per recovered ova/embryos were obtained in the control cows (80.7%), followed by CIDR®-1 (69.8%), and CIDR®-2 (61.4%) with a total average of 70.7% in all the treatment groups (Table 1).

In trial A, the mean ovulation rate and numbers of recovered ova/embryos were higher in cows treated with CIDRs® containing P_4 (CIDR®-1 and CIDR®-2) than the Control cows, but they were not significantly different (Table 2). Similar tendencies were observed in the mean ovulation rates

Table 1. Mean (± SEM) ovulation rate, number of embryos recovered and transferable embryos in superovulated cows per treatment group.

Treatments*	No. of cows (ovulations)	Ovulation rate/cow	No. of ova and embryos		
			recoverd	transferable	[%]
Control	15 (156)	10.4 ± 2.6	8.3 ± 2.9	6.7 ± 2.1	[80.7]
CIDR®-1	16 (182)	11.4 ± 1.9	10.6 ± 3.3	7.4 ± 1.3	[69.8]
CIDR®-2	15 (162)	10.8 ± 2.3	8.8 ± 3.0	5.4 ± 2.3	[61.4]
Total	46 (500)	10.9 ± 2.3	9.2 ± 3.1	6.5 ± 2.1	[70.7]

*Control : CIDR® without P_4 (placebo) inserted for 12 days.
CIDR®-1 : CIDR® containing P_4 inserted for 12 days.
CIDR®-2 : CIDR® containing P_4 inserted for 9 days, and replaced with a new CIDR® on day of the 12 day insertion period.

Table 2. Mean (± SEM) ovulation rate, number of embryos recovered and transferable embryos of superovulated cows in different seasons and years

Trials	No. of Cows (ovulations)	Ovulation rate/cow	No. of ova and embryos		
			recovered	transferable	[%]
A-Autumn 1991					
Control	5 (48)	9.6 ± 1.2	7.6 ± 1.5	7.0 ± 1.3	[92.1]
CIDR®-1	6 (80)	13.3 ± 2.0	10.8 ± 2.5	8.0 ± 1.9	[74.1]
CIDR®-2	6 (63)	10.5 ± 2.7	10.3 ± 3.4	5.0 ± 2.3	[48.5]
Total	17 (191)	11.2 ± 2.2	9.7 ± 2.7	6.6 ± 2.0	[68.0]
B-Spring 1992					
Control	5 (48)	9.6 ± 4.2	10.8 ± 4.3	7.6 ± 2.5	[70.4]
CIDR®-1	5 (60)	12.0 ± 1.8	15.0 ± 4.7	9.6 ± 1.6	[64.0]
CIDR®-2	4 (56)	14.0 ± 1.4	12.0 ± 3.2	8.3 ± 3.6	[69.2]
Total	14 (164)	11.7 ± 2.9	12.6 ± 4.3	8.5 ± 2.6	[67.5]
C-Autumn 1992					
Control	5 (60)	12.0 ± 1.9	6.6 ± 2.7	5.4 ± 2.5	[81.2]
CIDR®-1	5 (42)	8.4 ± 1.0	5.8 ± 1.4	4.6 ± 1.4	[79.3]
CIDR®-2	5 (43)	8.6 ± 1.6	4.4 ± 0.9	3.6 ± 0.5	[81.2]
Total	15 (145)	9.7 ± 1.6	5.6 ± 1.8	4.5 ± 1.5	[80.2]

* Descriptions for Table 2 are the same as in Table 1.

and numbers of recovered ova/embryos in trial B. However, in trial C the mean ovulation rates in cows treated with one and two CIDRs® containing P_4 (8.4 and 8.6, respectively) were lower than the control cows (12.0). Also, in trial A the highest (92.1%) and the lowest (48.5%) percentage of transferable embryos were obtained in the control and CIDR®-2 treatment groups, respectively, but they were not significantly different (Table 2). It was observed that in trial B, the mean numbers of recovered ova/embryos were higher than the number of ovulations (control: 10.8 vs 9.6 and CIDR®-1: 15.0 vs 12.0) (Table 2). Trial C showed a high total (80.2%) percentage of transferable embryos per recovered ova/embryos followed by trials A (68%) and B (67.5%) in all the 3 treatment trials (Table 2).

No significant differences were observed for both plasma P_4 and E_2 concentrations on the day of CIDR® insertion (day 0), the beginning of FSH injection (day 9), the injections of $PGF_2\alpha$ analogue (day 11), at the time of AI (day 13) and embryo recovery (day 20) (Fig. 2). However, on day 2 of CIDR® insertion, mean plasma P_4 significantly ($p<0.01$) varied among the three treatment groups (Control; 1.0±1.0, CIDR®-1; 2.8 ± 2.7, and CIDR®-2; 3.7 ± 2.7 ng/ml). Likewise, more than 1 ng/ml (1.4 ± 0.9) of plasma P_4 was obtained at the time of CIDR® removal (day 12) in CIDR®-2 and was significantly ($p<0.01$) higher than the other treatment groups (Control; 0.5 ± 0.4, CIDR®-1; 0.9 ± 0.5 ng/ml) but, both (day 2 and 12) of their E_2 concentrations were not significantly different among the groups.

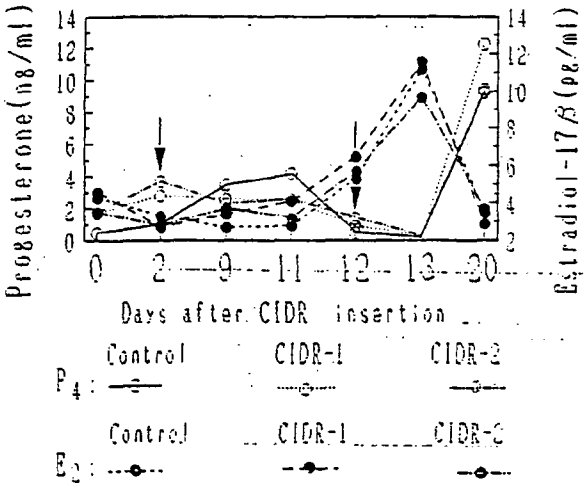

Fig. 2. Mean (± SEM) progesterone (P_4) and estradiol-17β (E_2) concentrations of superovulated cows per treatment group. ↓ : Arrows indicate significant ($p<0.01$) differences in P_4 levels among the groups.

Mean plasma P_4 and E_2 concentrations of each group per trial were shown in Fig. 3. No significant differences exhibited for both plasma P_4 and E_2 concentrations on day 0 and day 2 of CIDR® insertion, and at the time of AI (day 13) in all trials. At the beginning of the injection of FSH (day 9), both the plasma P_4 and E_2 levels showed significant ($p<0.01$) differences among the groups in trials B and C. The mean concentrations of plasma P_4 in the control cows (5.5 ± 2.4 ng/ml) and E_2 concentrations in CIDR®-1 (5.9 ± 3.9 pg/ml) in trial B, and the plasma P_4 of CIDR®-2 (0.9 ± 0.6 ng/ml) and E_2 of CIDR®-1 (1.2 ± 1.2 pg/ml) in trial C were significantly ($p<0.01$) different from the other treatment groups (Trial B, P_4 of CIDR®-1, 2.2 ± 1.4 and CIDR®-2; 3.0 ± 1.4 ng/ml, E_2 of Control; 3.5 ± 1.4 and CIDR®-2; 3.2 ± 1.2 pg/ml; Trial C, P_4 of Control; 1.6 ± 1.2 and CIDR®-1; 3.8 ± 4.8 ng/ml, E_2 of the Control; 1.8 ± 2.7 and CIDR®-2; 4.6 ± 2.7 pg/ml). The mean concentrations of plasma P_4 at the time of $PGF_2\alpha$ injection (day 11) were not significantly differed within the treatment groups. The plasma E_2 at the $PGF_2\alpha$ injection (day 11) of the control for both trials A and C (2.4 ± 0.6, 2.3 ± 1.2 pg/ml, respectively) were significantly lower ($p<0.01$) than the others (Trial A, CIDR®-1; 3.4 ± 1.3, CIDR®-2; 3.0 ± 0.8: Trial C, CIDR®-1: 2.7 ± 2.2, CIDR®-2; 3.0 ± 1.9 pg/ml), while in trial B the E_2 levels of CIDR®-1 (6.4 ± 3.6 pg/ml) was significantly ($p<0.01$) higher than the others (Control; 3.8 ± 2.0, CIDR®-2; 4.0 ± 1.7 pg/ml). At CIDR® removal (day 12), three treatment groups (Trial A; CIDR®-1 and CIDR®-2, and Trial B; CIDR®-2) have had more than 1 ng/ml P_4 concentrations (1.0 ± 0.5, 2.0 ± 0.9 and 1.1 ± 0.6 ng/ml, respectively) and those were significantly ($p<0.01$) higher than the other treatment groups (Trial A, Control; 0.7 ± 0.6: Trial B, Control; 0.4 ± 0.2 and CIDR®-1; 0.9 ± 0.5 ng/ml) and in trial A the P_4 concentration of CIDR®-2 was significantly higher than CIDR®-1. Their plasma E_2 levels were not significantly different among the groups. At embryo recovery (day 20), the mean plasma P_4 were not significantly different within the treatment groups. The mean plasma E_2 of the control and CIDR®-1 (0.9 ± 0.5, 0.7 ± 0.2 pg/ml, respectively) in trial C were significantly ($p<0.01$) lower than CIDR®-2 (4.5 ± 3.1 pg/ml), whereas in trial B those of the control and CIDR®-1

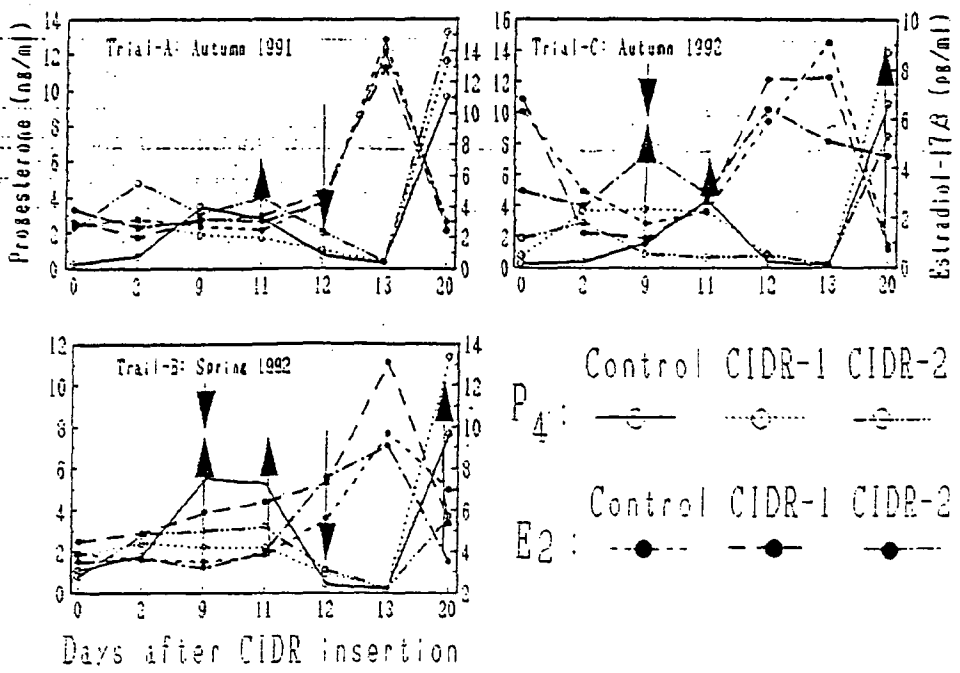

Fig. 3. Mean (± SEM) progesterone (P_4) and estradiol-17β (E_2) concentration of superovulated cows per trial. Arrows indicate significant ($p<0.01$) differences in the P_4 (↓) and E_2 (↑) levels among the groups.

(7.0 ± 3.1, 5.4 ± 3.0 pg/ml, respectively) were significantly ($p<0.01$) higher than CIDR®-2 (3.5 ± 0.6 pg/ml). The mean E_2 of the control was significantly higher than CIDR®-1 in trial B.

Discussion

It has been established that superovulation with the combination of IM injections of FSH and $PGF_2\alpha$ initiated on day 9 to 12 [5, 10, 12, 21–23, 25] of the estrous cycle of cows resulted in a greater yield of embryos. The present result of the control cows received CIDR® without P_4 (placebo) at days 2 to 4 of the estrous cycle showed that IM injection of FSH followed by $PGF_2\alpha$ treatment at the mid-cycle of the estrous cycle (day 11–13) had a high embryo recovery, but disagree to the findings of those initiated at the earlier stage (day 2–6) of the estrous cycle [6, 7, 24]. The present study has accorded with the report of Darrow *et al.* [22] that seasonality has no effect on the superovulatory response or fertility in dairy cows. A satisfactory mean of transferable embryos (Table 1: Total mean = 6.5 ± 2.1; Table 2: range=3.6 to 9.6) comparable to other experimental works [3, 5–7, 10, 12–14, 21–28] indicated that a satisfactory superovulatory scheme have been applied. Inspite that there were no statistical differences in the proportions of transferable embryos among the 3 treatment trials (Table 1), it appeared that control cows (80.7%) showed better than the CIDR®-1 (69.8%) and CIDR®-2 (61.4%). In addition, despite no statistical differences among the treatment groups, variability among individual animals to superovulatory response [5, 11, 16] and heterogeneous follicular development following superovulatory treatments [3] affected the high (92.1) and low (48.5) percentages of transferable embryos in trial A (Table 2). As rectal palpation of ovarian structures is not a precise measurement (6) and human factor had a vital role in it, underestimating the number of corpus luteum (CL) in cows with multiple ovulation was the reason for obtaining the mean (± SEM) average embryos (Control: 10.8 ± 4.3 and CIDR®-1: 15.0 ± 4.7) per flush in trial B (Table 2) which were higher than the mean (± SEM) number of CL (Control: 9.6 ± 4.2 and CIDR®-1: 12.0 ± 1.8) estimated. The mean (± SEM) CL palpated and embryo collected from trial B (Control and CIDR®-1 treatment groups were also greater than those reported by others for lactating and dry dairy cows [6, 21–23].

The present study confirmed the related works [4, 15, 29–35] that the drop of plasma P_4 and rise of

E_2 was the luteolytic activity of $PGF_2\alpha$. The mean hormonal concentrations in plasma of individual treatment group/trial shown in Fig. 3 were significantly ($p<0.01$) different in P_4 and E_2 on day 9, E_2 on day 11 and 20 while the total mean concentrations of plasma P_4 and E_2 did not differ on those days as shown in Fig. 2. Moreover, the total mean of plasma P_4 significantly differed among the treatment groups on day 2 and 12 (Fig. 2), but not its individual treatment group except on day 12 of trials A and B (Fig. 3). Considerable variability between individual animals in hormonal concentrations and timing of physiological events after the injections of gonadotrophic hormones [5, 15, 30] and heterogeneous follicular development following superovulatory treatments [3] may characterize the differentiation of plasma P_4 and E_2 levels of individual cow. No evidence exists for a significant correlation between P_4 and ovulation rate [5, 29, 30] at the time of AI (day 13). However, the different P_4 levels (Figs. 2 and 3) at CIDR® removal (day 12) were not entirely unexpected results and may have contributed to some of the differences in ovulation rate. Plasma samples in the present study were obtained only on the daily basis of day 0 and day 2 of CIDR® insertion, estrous cycle (day 9–13) and embryo recovery (day 20), respectively. But, when the blood collection is performed within the range of 2–24 h [4, 15, 31–35] and the subsequent monitoring of the ovarian structures with ultrasound device [4] were carried out, it may appear a result correlated with ovulation and embryo yield. This phenomenon is of clinical interest which may required further studies to determine the interrelation between the plasma levels at the time of CIDR® withdrawal and ovulation, and number of transferable embryos. Previous works stressed that FSH administration following PGF2α treatment does not affect luteolysis [4, 29] but interfere normal development of antral follicles and not effective in monitoring the viability of large dominant follicles or in improving the synchrony of return to estrus and ovulation [4]. The present study is consistent with the former but contradicts to the latter statement. Satisfactory results obtained in the present study is due to the effective use of CIDR® for synchronization of estrus for irregular cycling [14] or even in non-cyclic cows as reported by Broadbent *et al.* [18] and supported by Vargas *et al.* [36].

On the basis of the foregoing results, it may be concluded that the use of CIDR® combined with superovulation treatments (FSH+$PGF_2\alpha$) presents a satisfactory superovulatory regimen. However, this preliminary investigation on the use of CIDR® for superovulation is of encouraging results, further detailed systematic studies will be required before the overall effectiveness can be assessed.

Acknowledgments

The authors wish to express their thanks to the Managers of Kitami Center, Hokkaido Livestock Improvement Association, Kunneppu, Tokoro-gun, Hokkaido, Japan for the use of animals and facilities. To Carter Holt Harvey Plastic Products Ltd., of New Zealand and Mr. Y. Miyawaki, Surge Miyawaki Co. Ltd., the authors thank for the supply of CIDR® devices. Also, the authors thank to Dr. Y. Terawaki and Mr. Kieu Min Luk for the statistical assistance, and Messers S. Tamano and Y. Muto for the radioimmunoassay of blood samples.

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Québec 2004 Canada
23e Congrès mondial de buiatrie • Québec, Canada, 11-16 juillet 2004
23rd World Buiatrics Congress • Québec, Canada, July 11-16, 2004
23 Congreso Mundial de Buiatría • Québec, Canada, 11-16 de Julio 2004
23. Welt-Kongress für Buiatrik • Québec, Canada, 11.-16. Juli 2004

Factors influencing the success of embryo transfer in cattle

John F. Hasler[1]
Bioniche Animal Health USA, Inc.
Bogart, GA 30622

Introduction

Embryo transfer is one step in the process of removing one or more embryos from the reproductive tract of a donor female and transferring them to one or more recipient females. Embryos also can be produced in the laboratory via techniques such as in vitro fertilization (IVF) or somatic cell cloning. But the actual transfer of an embryo is only one step in a series of processes that may include some or all of the following: superovulation and insemination of donors, collection of embryos, isolation, evaluation and short-term storage of embryos, micromanipulation and genetic testing of embryos, freezing of embryos and embryo transfer.

Embryo transfer, first successfully accomplished by Walter Heape in 1890, started as a research tool and became a commercial enterprise in cattle in the early 1970s. The development of embryo transfer technology recently was reviewed historically very comprehensively by Betteridge (1). Commercial embryo transfer is now a large, international business.

Superovulation

There have been few improvements in the superovulation of cattle over the last 25 years. Illustrating this, it was recently shown that the average number of embryos recovered from superovulated cattle at Em Tran, Inc. was 4.6 from 248 donors in 1979 and 4.8 from 1485 donors 20 years later in 1999 (7). These data included all donors superovulated, whether or not they came into estrus or were rejected due to no palpable ovarian response at the time of embryo collection. A serious problem is that approximately 20% of donors produce no usable embryos.

In spite of the fact that embryo production per donor has not improved, there have been increases in embryo production per donor on a per unit time basis. This has been made possible largely through the use of intravaginal or subcutaneous progesterone-releasing devices. Superovulation can now be initiated following insertion of a progesterone-releasing device at any time of the estrous cycle. In addition, it has been clearly shown that donors do not benefit from having two estrous cycles between superovulations as was widely formerly believed. Donors are now repeatedly superovulated for a period of 1 to 2 years, every 40 days or less with very satisfactory results (7).

Dominant follicle removal has been shown to increase the number of embryos produced by superovulation in some studies but not others. Increased understanding of the processes of oocyte growth and maturation is essential to improving the efficiency of superovulation (8).

Embryo Recovery

Following the widespread adoption of non-surgical recovery, often referred to as flushing, of embryos in the mid 1980s, the procedures for recovering embryos have received little attention. Virtually all practitioners utilize Foley-type catheters with an inflatable cuff. Most practitioners opt for using a large volume (one to two liters) of flush fluid that is introduced by gravity flow, although opinions seem to be equally divided on uterine body versus horn flushing. For body flushing, the cuff is inflated just anterior to the cervix, allowing the uterine body and both horns to be flushed simultaneously. For each horn to be flushed separately, the catheter is inserted part way up one horn and then the other. Efficacy of embryo recovery appears similar for both approaches. In contrast, some practitioners achieve fine results by introducing a very small volume of medium with a syringe.

Traditionally, Foley catheters were composed of rubber or latex. Recently, several manufacturers have produced silicone catheters specifically designed for recovering embryos from cattle. These catheters have several advantages including the ability to withstand autoclave sterilization, cuffs that maintain a concentric conformation and multiple drainage ports.

Embryo Evaluation and Handling

Initially, the commercial embryo transfer industry primarily utilized simple media such as phosphate buffered saline supplemented with serum for flushing and storage of embryos. A number of companies now offer more complex media specifically designed for embryo transfer, although it remains to be proven that this has led to an improvement in success rates.

Evaluation of embryos is now relatively well standardized for both stage of development and quality based on definitions developed by the International Embryo Transfer Society. Several procedures for predicting the viability of embryos based on metabolism have been described. Currently, however, only microscopic morphology is used for evaluating embryos. Although morphology does not offer predictability on any given embryo, average pregnancy rates relative to embryo quality are highly predictive (6).

Embryo Transfer

Success rates with embryo transfer in many commercial situations are consistently high, often exceeding 70% pregnancy rates. In fact, when high quality fresh embryos are transferred into suitable recipients, pregnancy rates can average nearly 80% (6). Assuming technical competence on the part of the practitioner, the major factors influencing pregnancy rate are probably embryo quality and recipient suitability. Consequently, future increases in pregnancy rates beyond what is currently technically possible will probably be very incremental. For example, any change in technique involving an increase of 5 percentage points in pregnancy rates would be very important. Unfortunately, experimental proof that a specific treatment leads to such a small improvement involves very large number of transfers. Very few

academic institutions or commercial programs can afford to conduct experiments on the scale necessary to provide statistical significance.

Embryo quality is well known as a significant factor in pregnancy rate. However, practitioners have little choice regarding this variable when it comes time to transfer, whereas numerous variables related to recipients provide the opportunity for influencing pregnancy rate. Comparisons between different studies regarding recipient factors are not always legitimate. Recently, however, it was shown that pregnancy rates were similar among beef cows and heifers of *Bos taurus* breeds and dairy heifers (6), while a substantially lower pregnancy rate was achieved using dairy cows as recipients.

It has long been know that the degree of estrus synchrony between embryo and recipient is related to pregnancy rate. Several early studies seemed to indicate that synchrony was more critical in beef recipients than in dairy recipients. However, when beef and dairy recipients were compared in the same study (6), there was no difference in synchrony requirements. Also, it appears that 24 h plus or minus asynchrony between donor and recipient does not compromise pregnancy rate whether fresh or frozen-thawed embryos are transferred (6).

There has been a great deal of effort directed at identifying a hormone (progesterone, hCG, rbST, GnRH) or drug (banamine, clenbuterol) that improves pregnancy rate in embryo transfer recipients. These agents have been utilized in numerous studies directed at improving pregnancy rates in recipients without any clear, consistent improvement being demonstrated. Recently, the use of a low dose (400 IU) of eCG has resulted in improved pregnancy rates in embryo transfer recipients in several field trials.

Embryo Freezing

Largely as the result of pioneering work at Cambridge, embryos freezing of cattle embryos became a dependable and commercially viable tool in the early 1980s. Primarily utilizing glycerol as a cryoprotectant, the only disadvantage of this technology was that a microscope, specific thawing media and a trained embryologist were necessary at the time of thawing. Using ethylene glycol as a cryoprotectant instead of glycerol made possible the direct transfer of embryos directly from the straw in which they were frozen and provided a very significant improvement in the field of embryo transfer in the early 1990s. Pregnancy rates appear to be very similar between embryos frozen in glycerol and ethylene glycol. As a consequence, ethylene glycol is now the predominant cryoprotectant used in most commercial embryo transfer programs. Pregnancy rates resulting from transfer of frozen-thawed embryos are currently only approximately 10 percentage points lower than fresh embryos of similar quality.

IVF

In the early 1990s a number of embryo transfer businesses started offering IVF procedures on a commercial basis. This resulted largely from academic research breakthroughs in defining in vitro maturation conditions, capacitation procedures and temperature requirements for IVF. Commercial production of IVF-derived embryos became highly successful and as a result, many thousands of pregnancies were established, primarily under conditions involving in vitro culture with serum and coculture. Unfortunately, a significant number of pregnancies were characterized by early abortion, calving difficulties, perinatal deaths or calf abnormalities (5).

As a result, the demand for IVF services in North America has declined significantly. However, there is evidence that the use of semi-defined culture systems may result in an improvement in the percentage of normal pregnancies. In fact, it has been suggested that in the future, IVF procedures for embryo production may replace traditional embryo transfer involving superovulation and flushing (2,4).

Embryo Manipulation

The first successes in cloning cattle involved the division of embryos into two half or demi embryos. This technique can be accomplished either with the aid of a micromanipulator or by hand and results in both an overall increase in the number of calves produced from a group of embryos and also in the production of identical twins from some embryos. However, this technology is being utilized less frequently today than during the period when in first became technically feasible in the mid 1980s.

Another utilization of manipulation involves the removal of a few cells from embryos with the use of a micromanipulator and PCR analysis of sex or the presence of certain genotypes. In addition to the need for relatively sophisticated and expensive equipment, this technology requires a high level of skill and consequently has not been widely adopted by the commercial embryo transfer industry.

Cloning of adult cattle by the transfer of somatic cell nuclei is an area that is currently receiving enormous attention in the press and in academic research laboratories. The actual commercialization of cattle cloning is proceeding on a somewhat limited scale (3). In the USA, continued growth of cattle cloning is dependent on a clearly defined policy decision by the Food and Drug Administration.

Biosecurity

There has been little attention focused on the relationship between disease and embryo transfer on a domestic basis within countries. As the international trade in frozen embryos grew rapidly during the 1980s, however, this subject received a good deal of attention and very specific protocols have been developed for the production and handling of embryos destined for movement between countries. These protocols have proven quite effective and there are no indications that any identified microbes have been transported internationally in association with embryos. The protocols in place do not necessarily apply to embryos produced by in vitro procedures and more research is necessary to develop effective sanitary regulations for the production of in vitro, cloned and transgenic embryos (9). In light of recent international outbreaks of foot and mouth and BSE, it is highly likely that the use of media containing no products of animal origin will be mandated for the handling and freezing of all cattle embryos.

Abstract

Embryo transfer in cattle has grown into a mature, international business with high success rates. Future improvements will involve small incremental changes that are difficult to prove experimentally.

Résumé
Le transfert embryonnaire est rendu à un niveau commercial mature et qui atteint des niveaux de succès élevés. Les améliorations que connaîtra cette technologie seront minimes et difficiles à démontrer expérimentalement.

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[1]Corresponding Address: John F. Hasler, 427 Obenchain Rd., Laporte, CO 80535, jfhasler@viawest.net

Superovulation in Perspective

(Revised December 2, 2002)

BIONICHE Animal Health is pleased to provide an overview of research in the field of embryo transfer. In today's fast paced workplace, keeping up with the literature can be a challenge. BIONICHE Animal Health hopes that this service will be of benefit to you.

Please contact Bioniche Animal Health if you have any questions relating to this article.

Reuben J. Mapletoft,[1,2] Kristina Bennett Steward,[2] and Gregg P. Adams[1]

[1] *Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5B4, and*

[2] *BIONICHE Animal Health, Belleville, ON K8N 5J2, Canada*

ABSTRACT

Variability in superovulatory response continues to be one of the most frustrating problems with embryo transfer in cattle. The removal of LH from pituitary extracts has tended to reduce variability in response, and several studies involving the use of the purified porcine pituitary extract, Folltropin®-V are reviewed. The major source of variability in superovulatory response in cattle is the status of ovarian follicles at the time of initiation of gonadotrophin treatments. Data support the benefits of initiating gonadotrophin treatments at the time of emergence of a follicular wave. Incorporation of techniques designed to control follicular wave dynamics, such as follicular ablation, or treatment with estradiol/progesterone, have reduced the variability caused by treating cows at different stages of follicular development, and at the same time improved response by taking advantage of endogenous recruitment and selection mechanisms. New protocols offer the convenience of being able to initiate gonadotrophin treatments quickly and at a self-appointed time, without the necessity of estrus detection and without sacrificing response. Methods can be used for repeated superstimulation of donor animals at 25 to 30 day intervals, without regard to estrus detection or stage of the estrous cycle, and without compromising embryo production.

Superstimulation / gonadotrophin, / FSH / LH / follicular waves / embryo transfer / cattle

1. INTRODUCTION

The objective of superstimulatory treatments in the cow is to obtain the maximum number of fertilized and transferable embryos with a high probability of producing pregnancies [6]. Wide ranges in superovulatory response and embryo yield have been detailed in several reviews of commercial embryo transfer records. In a report of 2048 beef donor collections, a mean of 11.5 ova/embryos with 6.2 transferable embryos were collected from each cow [27]. However, variability was great in both the superovulatory response and embryo quality; 24% of the collections did not produce viable embryos, 64% produced fewer than average numbers of transferable embryos and 30% yielded 70% of the embryos. Embryo recovery from 987 dairy cows yielded slightly fewer ova/embryos and there was similar variability in response among animals [25]. The high degree of unpredictability in superovulatory response creates problems affecting both the efficiency and profitability of embryo transfer programs [24].

Variability in ovarian response has been related to differences in superovulatory treatments, such as gonadotrophin preparation, batch and total dose, duration and timing of treatment, and the use of additional hormones in the superovulatory scheme. Additional factors, which may be more important sources of variability, are inherent to the animal and its environment. These factors may include nutritional status, reproductive history, age, season, breed, ovarian status at the time of treatment and the effects of repeated superovulation. While considerable recent progress has been made in the field of bovine reproductive physiology, factors inherent to the donor animal which affect superovulatory response are only partially understood. The purpose of this review is to address practical aspects of bovine superovulation with a view to simplifying superstimulatory procedures, improving responses and reducing variability; in the interest of space, reference to review articles will be done as much as possible.

2. GONADOTROPINS AND SUPEROVULATION

Factors associated with the administration of exogenous gonadotrophins affecting superovulatory response include source, batch and biological activity of the gonadotrophin [36]. We have investigated the biological activity of gonadotrophins and the effect that FSH and LH activities in gonadotrophin preparations have on the superovulatory response in the cow. We have also investigated the use of the highly purified porcine pituitary extract (Folltropin®-V; BIONICHE Animal Health, Bellville, ON, Canada) [7, 20]. Pertinent research on the biological activity of gonadotrophins and how this affects superovulatory response in the cow will be reviewed.

Three different types of gonadotrophins have been used to induce superovulation in the cow; gonadotrophins from extracts of porcine or other domestic animal pituitaries, equine chorionic gonadotrophin (eCG) and human menopausal gonadotrophin (hMG) [5, 36]. Prostaglandin (PGF) or its analogues have been used for the induction of luteolysis in a superstimulatory regimen, to allow for precise timing of onset of estrus and of ovulation. The biological half-life of FSH in the cow has been estimated to be 5 h or less so it must be injected twice a day to successfully induce superovulation [33]. The usual regimen has

been 4 or 5 days, twice daily treatments of FSH with a total dose of 28 to 50 mg (Armour) of a crude pituitary extract (FSH-P) or 400 mg NIH-FSH-PI of the purified pituitary extract, Folltropin®-V. Forty-eight or 72 h after initiation of treatment, PGF is injected to induce luteolysis. Estrus occurs in 36 to 48 h, with ovulation 24 to 36 h later.

Equine chorionic gonadotrophin is a complex glycoprotein with both FSH and LH activity [35]. It has been shown to have a half-life of 40 h in the cow and persists for up to 10 days in the bovine circulation; thus it is normally injected once followed by a PGF injection, 48 h later [18]. The long half-life of PMSG causes continued ovarian stimulation, unovulated follicles, abnormal endocrine profiles and reduced embryo quality [32, 34, 41]. These problems have been largely overcome by the intravenous injection of antibodies to eCG at the time of the first insemination, 12 to 18 h after the onset of estrus [18, 21]. Recommended doses of eCG range from 1500 to 3000 IU, with 2500 IU by intramuscular injection commonly chosen.

Monniaux et al. [33] treated a group of cows with 2500 IU eCG and another with 50 mg (Armour) FSH-P and observed that ovulation rate and the percentage of cows with more than 3 transferable embryos was slightly higher with FSH-P than eCG. Although these results were in agreement with those of Elsden et al [19] others have found no differences between pituitary FSH extracts and eCG [5, 22, 30]. Endocrine studies have revealed that eCG-treated animals more frequently had abnormal LH and progesterone profiles than did the FSH-treated cows [23, 32]. These were associated with reductions in both ovulation and fertilization rate [15]. In a study of cows repeatedly superstimulated at 60 to 90 day intervals over 1 year, we found no differences in superovulatory response between two different pituitary extracts (Folltropin®-V or FSH-P) and eCG with or without a monoclonal antibody to eCG (Neutra-PMSG; Intervet, Boxmeer, Holland) administered at the time of the first insemination [30]. However, numbers favored Folltropin®-V and eCG with with Neutra-PMSG. Others have made similar observations [22].

Although folliculogenesis in mammals requires both FSH and LH, there is considerable variability in FSH and LH content of crude gonadotrophin preparations. Radioreceptor assays and in vitro bioassays have revealed variability in both the FSH and LH activity of eCG, not only among pregnant mares, but also between bleedings in the same mare at different times during gestation [35]. We have also examined the effects of the FSH/LH ratio of eCG on superovulatory responses with immature rats and found that there was a positive correlation between the ratio of FSH/LH activity and superovulatory response. Lower ratios of FSH/LH activity appeared to reduce ovulatory response in rats and additional LH, when added to eCG reduced superovulatory response in cows [35, 36].

Purified pituitary extracts with low LH contamination have been reported to improve superovulatory response in cattle. Chupin et al. [16] superstimulated three groups of dairy cows with an equivalent amount of 450 μg pure pFSH and varying amounts of LH, and showed that the mean ovulation rate and the number of recovered and transferable embryos increased as the dose of LH decreased. They observed that as LH activity increased, the dose of FSH required to induce an acceptable response also increased. It

has been suggested that embryo quality may be adversely influenced by high LH levels during superstimulation due to premature activation of the oocyte [34].

We have completed several experiments with the LH-reduced Folltropin®-V utilizing several different total doses, ranging from 100 to 900 mg of NIH-FSH-P1 activity [5, 20]. There was no evidence of detrimental effects of dose on embryo quality. Ovulation rates continued to increase to 400 mg NIH-FSH-P1 (40 mg Armour) and did not increase beyond that dose. At the same time fertilization rate and transferable embryo rate remained constant throughout the dose range used. On the other hand, doubling the dose of LH-rich preparations (FSH-P or hCG) resulted in significantly reduced fertilization rates and percentages of transferable embryos [5]. Collectively, data support the hypothesis that the detrimental effects of high doses of pituitary gonadotrophins on ova/embryo quality is due to an excess of LH.

Recently, we investigated the long-term safety of Folltropin®-V in a retrospective study involving 1949 donor cows and their offspring i.e., second and third generation donor cows which were a result of superovulation and embryo transfer. Reproductive safety was examined by calculating the number of viable embryos collected from each cow and the number of normal calves born to cows that had been previously superstimulated with Folltropin®-V. Embryological safety was measured by the number of live calves produced from superovulation and embryo transfer using Folltropin®-V. The main data set examined all available records with respect to treatment number, number treatments in a sequence, the status of mother/donors and whether they were a product of embryo transfer, the number of known calves produced from embryo transfer and the number of calves born naturally to embryo donors. A smaller data set was based on known family relationships from four generations for the same end-points. Statistical analyses, based on analyses of variance, revealed no significant difference among the observed variables (numbers of embryo recovery, calves by embryo transfer, natural born calves etc.) as a consequence of the independent variables. We concluded that there was no evidence of adverse effects of treatment, or repeated treatment of donor cows with Folltropin®-V, on reproductive performance, embryo production or resulting offspring.

Although it is generally believed that some LH is required for successful superovulation, endogenous LH levels may be adequate. Looney et al. [28] reported that recombinantly produced bFSH induced high superovulatory responses without the addition of exogenous LH. In addition, fertilization rates exceeded 95% and viable embryos rates exceeded 85%. These data suggest that LH is not needed in superovulatory preparations and that embryo quality may be superior with pure FSH. The very high fertilization rates and transferable embryo rates in the absence of exogenous LH tend to suggest that administration of LH, at any dose, may be detrimental to embryo quality.

An experiment was designed to determine the effects of exogenously administered LH on superovulatory response in *Bos taurus* cattle [43]. Cross-breed beef cows were randomly placed into one of four treatment groups to be superstimulated with a total dose of pFSH equivalent to 400 mg NIH-FSH-P1 over 4 days. Cows in Group I received a standard porcine pituitary extract much like FSH-P (100% LH), whereas cows in Group II

received a preparation with approximately 68% LH removed (32% LH), cows in Group III received a preparation with approximately 84% LH removed (16% LH - equivalent to Folltropin®-V), and cows in Group IV received a preparation with 98% LH removed (Pure FSH). Superovulatory responses clearly divided these cows into two distinct groups (Table I); those with high LH (Groups I and II) and those with low LH (Groups III and IV). Overall, there were more ovulations, ova/embryos collected ($P<0.05$), and there tended to be more fertilized ova ($P<0.07$) in the two groups with the least LH (Groups III and IV). With the doses used in this experiment, there was no affect of LH on ova/embryo quality. Results demonstrate that LH within FSH preparations affects superovulatory response and that the maximum acceptable level of LH would appear to be between 15 and 20%.

Table I. Superovulatory response of *Bos taurus* cows superstimulated with FSH (400 mg NIH-FSH-P1) and varying amounts of LH [43].

Group	n	CL	Total	Ova/Embryos Fert (%)	Trans (%)
I (100% LH)	21	10.2^{a}	7.3^{a}	5.3^{c} (73)	4.0 (55)
II (32% LH)	20	11.1^{a}	6.4^{a}	4.6^{c} (72)	3.9 (61)
III (16% LH)	20	15.6^{b}	13.6^{b}	9.7^{d} (71)	7.7 (57)
IV (Pure FSH)	20	17.2^{b}	13.2^{b}	8.3^{d} (63)	5.5 (42)

Means with different superscripts are different (ab - $P<0.05$; cd - $P<0.07$).

In yet another experiment involving Brahman-cross (*Bos indicus*) heifers superstimulated with 400 mg NIH-FSH-P1 containing 100%, 16% or 2% LH, Tribulo et al. [42] reported that the more purified preparations caused the higher superovulatory response (Table II). Overall, the most purified preparation (Group III) induced more CL and tended to result in more ova/embryos and fertilized ova when compared to the least purified preparation (Group I). The intermediate preparation (16% LH; group II) induced an intermediate response. However, there were obvious seasonal effects. Responses with pure FSH and 16% LH were superior to the crude extract (100% LH) during summer months, but only the pure FSH was more efficacious during winter months.

Table II. Superovulatory responses of Bos indicus heifers, superstimulated with FSH (400 mg NIH-FSH-P1) and varying amounts of LH [42].

	Summer			Winter			Overall	
Group n	CL	TO/E FO	n	CL	TO/E FO	n	CL	TO/E FO
I (100% LH) 3.9	13	8.5 4.7	4.2	14	3.7[a] 4.4	3.4	27	6.0[a] 4.6
I (16% LH)* 4.0	12	19.2 9.6	7.0	15	5.9[a] 1.6	0.8	27	11.7[ab] 5.8
III (Pure FSH) 6.8	14	16.5 7.0	5.7	15	19.4[b] 10.6	8.3	29	18.1[b] 8.5

ab - Means within a column with superscripts not in common are different ($P<0.05$)
* Group II differed between summer and winter ($P<0.05$).

These results would appear to contradict the findings of Page et al. [39] who reported that superovulation and embryo quality in Holstein heifers was not affected by LH levels in cool weather; whereas a low LH preparation (Folltropin®-V) yielded more CL and significantly more fertilized ova and transferable embryos during heat stress. It becomes apparent that stress is the common factor. *Bos taurus* breeds likely find summer heat stressful, whereas *Bos indicus* breeds likely find winter temperatures stressful. In either case, the more purified extracts resulted in greater superovulatory responses during conditions of stress.

We have also investigated the use of Folltropin®-V as a single bolus injection for superstimulation of cattle. A single subcutaneous injection of Folltropin®-V at a dose equivalent to 400 mg NIH-FSH-P1 resulted in a superovulatory response equivalent to that of a 4 day, twice daily intramuscular treatment regimen [10]. During the course of these studies, it was found that a more consistently high superovulatory response occurred when the subcutaneous injection was made behind the shoulder as opposed to in the neck region. We have since found that splitting the single subcutaneous dose (Day 0 - 75%; Day 2 - 25%) improved results in cows with little subcutaneous fat [29], and we have preliminary results suggesting that the ischiorectal fossa may be an alternative site for a single injection of FSH [17]. In fact, anything that results in increased absorption of FSH (eg. intramuscular injection or injection in the neck region of lean cows) resulted in a reduced superovulatory response. Although Folltropin®-V has been reported to have more than 80% of LH removed, there may be sufficient LH remaining to result in an over-dose when administered in a single bolus injection or when absorption rate is increased.

A single bolus subcutaneous injection of Folltropin®-V has much to offer superstimulatory treatment protocols, especially when twice daily treatments may result in stress which may suppress superovulatory response. In one study involving *Bos indicus* heifers, a single subcutaneous injection of Folltropin®-V resulted in a significantly greater superovulatory response than a twice daily, four-day treatment schedule [10]. We attributed the difference to the stress associated with twice daily treatments and handling.

When comparing experiments, route of administration must also be considered [reviewed in 5]. We have observed that twice daily intramuscular injections of Folltropin®-V resulted in a significantly higher superovulatory response than twice daily subcutaneous injections. We have also demonstrated that a single intramuscular injection resulted in higher circulating FSH levels than did a single subcutaneous injection [5]. However, the subcutaneous injection resulted in a more prolonged increase in FSH levels and a significantly improved superovulatory response [10].

Individual studies often show little or no difference in results among the various gonadotrophins used for superovulation in the cow. It is also obvious that breed, environment, nutrition and the individual animal response are factors which complicate ovarian stimulation. The role of stress has not been well documented, and more studies are required to understand the stress-cortisol-cytokine-hormone effects on reproductive performance and superovulation.

3. ANIMAL INFLUENCES ON SUPEROVULATION

With a better understanding of ovarian function has come a greater capability of controlling it. Our expanding knowledge of the roles of the CL and follicular waves in the bovine estrous cycle has resulted in renewed enthusiasm about the prospects of precise synchronization of estrus and ovulation. The intention of the following discourse is to provide an overview of normal ovarian events in cattle, and to discuss how these events impact on the effectiveness of superstimulation regimens. We hypothesized that ovarian response to exogenous stimulation is contingent upon the physiologic status of the ovaries at the time of superstimulation.

3.1. Ovarian Follicular Wave Dynamics

It has been shown that greater than 95% of bovine estrous cycles are composed of either two or three follicular waves [reported by Adams, Fortune, Ginther, Roche and Boland, and others; reviewed by Adams in 2]. Single-wave cycles have been reported in heifers at the time of puberty and in mature cows during the first interovulatory interval after calving. Four-wave cycles are observed occasionally in *Bos indicus* cattle [40]. The proportion of animals with two- versus three-wave cycles varies among reports; some report a majority of two-wave cycles and others report a majority of three-wave cycles while others have observed a more even distribution [reviewed in 2]. Although the subject has not been systematically studied, there does not appear to be a clear breed- or age-specific preference for one follicular wave pattern over the other, nor is there any

apparent difference in fertility [4]. In a study of the effects of nutrition on follicular dynamics, cattle fed a low energy ration had a greater proportion of three-wave cycles than those fed higher energy rations [37]. Preliminary data collected from 9 heifers during their first 2 years suggest that the pattern is repeatable within individuals (Adams, unpublished). In another study in *Bos indicus* cattle, four of 25 cows had four follicular waves per cycle; one cow changed from four waves in the spring to three waves in the autumn. The evolutionary reason for a two- or a three-wave cycle, or indeed for the wave-like pattern itself, is unclear; however, the differences in wave patterns are distinct and they have clear implications regarding ovarian synchronization and superstimulation.

Simply put, the wave pattern of follicular development refers to periodic, synchronous growth of a group of antral follicles. In cattle, follicle wave emergence is characterized by the sudden (within 1 to 2 days) growth of more than 20 small follicles that are initially detected by ultrasonography at a diameter of 3 to 4 mm [2]. For about 2 days, growth rate is similar among follicles of the wave, then one follicle is selected to continue growth (dominant follicle) while the remainder become atretic. In both two- and three-wave estrous cycles, emergence of the first follicular wave occurs consistently on the day of ovulation (day 0). Emergence of the second wave occurs on day 9 or 10 for two-wave cycles, and on day 8 or 9 for three-wave cycles. In three-wave cycles, a third wave emerges on day 15 or 16. Successive follicular waves will remain anovulatory until luteolysis occurs. The dominant follicle present at the onset of luteolysis will become the ovulatory follicle, and emergence of the next wave is delayed until the day of ovulation. The CL begins to regress earlier in two-wave cycles (day 16) than in three-wave cycles (day 19) resulting in a correspondingly shorter estrous cycle (20 days vs 23 days, respectively). Hence, estrous cycle length may provide a clue to numbers of follicular waves that a given cow has within each cycle.

3.2. Role of gonadotropins in follicular wave development

The mechanism involved with follicular wave dynamics is based on differential responsiveness of the ovary to FSH and LH [2]. Periodic surges in circulating concentrations of FSH are responsible for eliciting follicular wave emergence; hence, cows with two-wave cycles have two FSH surges and three-wave cycles have three surges [3]. Circulating FSH is subsequently suppressed by negative feedback by estradiol and inhibin from the emerging follicles and the following nadir in FSH effectively prevents new wave emergence. The transient rise in FSH permits sufficient follicular growth so that some follicles acquire LH responsiveness which allows survival without FSH. At the time of follicle selection, 2 or 3 days after wave emergence, FSH is declining rapidly. The follicle destined to become dominant apparently acquires receptors for LH and has the competitive advantage over follicles destined to become subordinate. However, LH responsiveness and the ability to become a dominant follicle likely represents a quantitative rather than an absolute difference between follicles in a wave. Subordinate follicles can become dominant if the original dominant follicle is removed or if exogenous FSH is supplied [2]. Further, the competition for LH among multiple dominant follicles (i.e., superstimulated with FSH) is apparent by the smaller maximum diameter attained compared to single dominant follicles. Continued suppression of LH as

a consequence of luteal-phase progesterone secretion causes atresia of the dominant follicle, and FSH is again allowed to surge. This surge has no effect on the dying dominant follicle, but is responsible for eliciting the emergence of the next wave. The ovarian cycle then repeats itself. Relief from progestational suppression (i.e., luteolysis) allows LH pulse frequency to increase, permitting further growth of the dominant follicle and dramatically higher circulating concentrations of estradiol, which results in a surge of LH followed by ovulation.

The conventional protocol of initiating ovarian superstimulation during mid-cycle (8 to 12 days after estrus) was arrived at empirically, but studies in which a lesser response to superstimulatory treatments initiated early in the estrous cycle (2 to 6 days after estrus) vs later (9 to 11 days after estrus) validated the convention [22, 26]. The reason for the relative success of the conventional approach may be explained by what we now understand about follicular dynamics.

We hypothesized that superstimulatory response would be greater if treatment was initiated before selection of a dominant follicle. In an initial study, recombinant bFSH given to heifers before the time of selection (day 1, ovulation = day 0) resulted in more ovulations than that given after the time of selection (day 5) of the dominant follicle of Wave 1 [1]. A subsequent study was done to determine if exogenous FSH given at the expected time of the endogenous wave-eliciting FSH surge had a positive effect on the superstimulatory response [38]. The endogenous surge in FSH was expected to peak 1 day before wave emergence, so superstimulatory treatments were initiated on the day before, the day of, or 1 or 2 days after wave emergence. Significantly more follicles were recruited and more ovulations occurred when treatment began on the day of, or the day before, follicular wave emergence.

In a direct comparison between waves, results of another study did not reveal any difference in the number of large follicles recruited, the number of ovulations induced, or the number of ova/embryos recovered in heifers in which superstimulation was initiated on the day of emergence of Wave 1 or Wave 2 [reviewed in 1]. Consistent with the previous study [38], when treatment was initiated $\geq$1 day after wave emergence, the superstimulatory response was reduced. These data suggest that superovulation may be induced with equal efficacy when treatment is initiated during the first or second follicular waves, and that the superstimulatory response is enhanced if treatment is initiated at the time of wave emergence.

Based on duration of the developmental phases of the dominant follicle in two-wave and three-wave interovulatory intervals, the probability at any given time that the dominant follicle is not functionally dominant is approximately 30% (6 of 20 days) for two-wave heifers and 35% (8 of 23 days) for three-wave heifers. More importantly, only 20% (4 or 5 days) of the estrous cycle is available for initiating treatment at the time of follicular wave emergence. Therefore, 80% of the cycle is not conducive to an optimal superovulatory response. To obviate these problems, studies have been done to determine if superstimulation subsequent to elective induction of follicular wave emergence could be used with equal efficacy to the conventional protocol.

One approach involved transvaginal ultrasound-guided follicle ablation to synchronize wave emergence among heifers at random stages of the cycle followed by the insertion of a progestogen implant and treatment with Folltropin®-V 1 day after ablation, and PGF 48 and 60 h later [9]. Non-ablated control heifers were given Folltropin®-V 8 to 12 days after estrus. Combined over two experiments (Table III), there was no difference in the superovulatory response between the ablated and non-ablated groups. In another study, Bungartz and Niemann [13] obtained a significantly higher superovulatory response when the dominant follicle was ablated 2 days before initiating gondadotrophin treatments. More recently, we have shown that ablation of the two largest follicles at random stages of the cycle will ensure that the dominant follicle is removed and a new wave will emerge 1 to 2 days later [8].

Another approach to the synchronization of follicular wave emergence for superovulation involves an injection of 5 mg estradiol-17ß after the insertion of a progestogen implant, followed by the administration of Folltropin®-V beginning 4 days after estradiol treatment [11, 12]. PGF was given 48 h after Folltropin®-V treatment was initiated and the progestogen implant was removed 12 h after PGF treatment. Control heifers were given the same dose of Folltropin®-V between 8 and 12 days after estrus. Combined over two experiments (Table III), the superovulatory response in the estradiol-treated groups was equivalent to that of the control groups.

Table III. Response in control heifers superstimulated between days 8 and 12 of the cycle compared to synchronization of wave emergence by follicle ablation or progestogen + estradiol (P+E) [9, 11].

	Ablation-induced wave synchrony			Steroid-induced wave synchrony	
	Control	Ablation		Control	P+E
No. of heifers	35	60		52	56
CL	22.9	18.6		23.7	24.3
Total ova/embryos	10.1	9.8		12.3	12.4
Fertilized ova	7.3	7.8		7.9	9.3
Transferable embryos	5.4	5.6		4.9	5.2

Our preferred approach to the synchronization of follicular wave emergence for superstimulation involves an injection of 5 mg estradiol-17ß plus 100 mg progesterone at the time of CIDR-B (BIONICHE Animal Health) insertion followed by Folltropin®-V given as a single or multiple dose beginning 4 days after estradiol treatment [11, 12]. PGF is given 48 h after Folltropin®-V treatment is initiated and the CIDR-B is removed 12 h later. Combined over several experiments, the superovulatory response in the estradiol-treated groups has been equivalent to or greater than that of control groups superstimulated on days 8 to 12 of the cycle. In a more recent experiment, we compared synchrony of follicular wave emergence and superovulatory response after treatment of

norgestomet-implanted cows with estradiol-17ß or estradiol valerate [31]. Follicular wave emergence occurred on days 3 or 4 (mean = 3.6 days) in all 37 cows treated with estradiol-17ß while follicular wave emergence occurred between days 3 and 6 (mean = 5.7 days) in 68% of estradiol valerate-treated cows. Superovulatory response and total ova/embryos collected were also greater in the estradiol-17ß-treated group. Data suggest that the greater synchrony of follicular wave emergence following treatment with estradiol-17ß and progesterone provided an advantage for the elective induction of superovulation. In another study, a dose of 1 mg of estradiol benzoate was as efficacious as 5 mg estradiol-17ß in synchronizing follicular wave emergence on day 4, whereas a dose of 5 mg estradiol benzoate resulted in a mean of 5 days with more variability [14]. Unfortunately, we have not investigated synchrony of follicular wave emergence following treatment with a reduced dose of estradiol valerate. In any case, these studies demonstrate that elective induction of follicle wave emergence offers the advantage of initiating superstimulatory treatment at a time that is optimal for follicle recruitment. Thus, the full extent of the estrous cycle is available for superstimulation and the need for detecting estrus or ovulation and waiting 8 to 12 days to initiate gonadotropin treatments is eliminated.

It is noteworthy that in studies involving superstimulation coincident with wave emergence, the response to a single bolus injection of Folltropin®-V was as good or better than the response to a multiple injection scheme. The nadir between FSH surges is responsible for preventing the emergence of a new wave; provision of exogenous FSH during the period of the FSH nadir may result in "break through" growth of small follicles prior to the time of expected new wave emergence (i.e., effects of dominant follicle suppression were overcome by FSH) [reviewed in 1, 2]. This may explain how large doses of exogenous FSH in conventional superstimulation schemes can overwhelm the endogenous rhythm and mask the wave effect. If superstimulatory treatment is given for a long enough period, follicle recruitment will become apparent, regardless of follicular wave status at the time of gonadotropin treatment. However, asynchronous recruitment may result in more variability in ovarian follicular response, and in the quantity and quality of oocytes and embryos collected.

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